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Symbiotic interrelationships of a pair of mesophilic and thermophilic bacilli

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SYMBIOTIC INTERRELATIONSHIPS OF A PAIR OF MESOPHILIC AND THERMOPHILIC BACILLI

by

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INTRODUCTION

Bacteriologists have found that pure cultures of microorganisms have a narrow optimum growth temperature range, usually ± 2-3C about their mean optimum growth temperature. The reported pure bacterial cultures which can grow over a temperature range of 30-70C, when investigated more thoroughly, have been shown to consist of thermoduric mesophiles and thermophiles, each of which grows in its preferred temperature range to give an appearance of unrestricted growth of a single organism over a wide temperature range.

Quinn (1949) studied such a culture in which a mesophile and thermophile grew symbiotically and digested cellulose at 65C. In this mixed culture, (C & E), he noted two distinct morphological types which were isolated by plating at (a) room temperature, 23-25C, in the case of culture E, and (b) 65C in the case of culture C. Neither of these organisms would digest cellulose alone, with typical orange chromogenesis, but when grown together at 65C, they digested cellulose vigorously. Thus, there apparently exists an ecological relationship between the mesophile and thermophile.

The most interesting problem posed by organisms capable of growth at 65C is a physiological one. How are these

microbes able to live and grow at temperatures so high that many proteins are coagulated and the existence of life appears as a biochemical anomaly? According to Allen (1953), the external environment of the cells, structural features of the organisms including structural modifications on a molecular level, and dynamic effects of metabolism in preventing or repairing thermal damage, all play a part in thermophily.

Koffler (1957) posed the question that if rapid synthesis really is the key to the problem of life at high temperatures, why can't mesophiles grow at 70C? Theoretically, an organism should be from 16 to 81 times as active at 70C as it is at 30C, as long as denaturation of essential molecules does not become the dominant reaction.

If a mesophile is capable of growth at 65C in nature, only when it is in association with a thermophile, then the existence of complex factors would logically seem to be involved. Since it was felt that symbiosis undoubtedly involves many interactions between the symbionts, research was directed toward the elucidation of several postulated factors which are coordinated in the function of this particular biological system.

REVIEW OF LITERATURE

Allen (1950, 1953) studied the intrinsic characteristics of thermophiles and concluded that these organisms can live at high temperatures because they can synthesize enzymes and other cellular constituents faster than these are destroyed by heat. In order to account for this great synthetic capacity at high temperatures, it has been postulated that these organisms have a higher temperature coefficient of enzyme synthesis than mesophiles. Since it has been shown that some thermophilic bacteria can grow at high temperatures without having notably thermostable respiratory enzymes, it is evident that the ability of thermophiles to counteract the destructive effects of heat must be important in permitting growth at thermophilic temperatures. It can be shown readily that even in those thermophilic bacteria which contain thermostable enzymes, cell material is destroyed during growth at high temperatures, and that active metabolism is necessary to keep the proportion of viable cells at a high level.

Studies involving growth requirements of thermophilic bacilli have suggested the fastidiousness of these organisms, including a definite methionine requirement (Baker *et al.*,

1960) and the necessity of nitrate ion in supporting sporulation (Dahl, 1955). Many reports in the literature dealing with the effect of temperature on the growth requirements of microorganisms have shown that as the incubation temperature is increased there is an increase in the growth requirements of the particular organism under study. The explanation of these findings is that at the higher temperature the enzyme(s) responsible for the synthesis of a particular required metabolite(s), say compound X, undergoes thermal inactivation and thus the organism requires an exogenous source of compound X before growth can take place. Campbell and Williams (1953) studied the nutritional requirements of facultative and obligate thermophiles and found that these organisms fell into three distinct groups: (a) those with no nutritional differences in growth requirements regardless of temperature, (b) those with additional requirements as the temperature was increased, and (c) those with additional requirements as the temperature was decreased. They have proposed the following respective explanations for the above phenomena: (a) the gene necessary for production of the enzyme responsible for the synthesis of X has been inactivated, and an exogenous source of X must be supplied,

(b) the gene responsible for the synthesis of X rather than being inactivated has become modified so that it now produces an enzyme with the same specificity, but altered physically in a manner which lead to rapid inactivation at higher temperatures, and (c) the gene responsible for the synthesis of X was not inactivated but modified to produce an enzyme dependent upon a high temperature for activation.

Georgi et al. (1951, 1955) isolated 'thermal' malic dehydrogenase, cytochrome oxidase, aphyrase, aldolase, cytochrome b, and succinoxidase from the 'red fraction' of Bacillus stearothermophilus. All of these enzymes were found to be stable to heat inactivation at 65C, the optimum growth temperature for the thermophilic organism. Further studies on thermal enzymes have been conducted by Militzer and Tuttle (1951), Thompson (1961), and Downey (1962). Militzer and Burns (1954) concluded that the factors responsible for the heat stability of thermal enzymes were the substrate, magnesium, and oxygen. These findings suggest that several methods may be employed by thermophiles for protecting themselves against the destructive effects of high temperatures. The organisms seem to make use of soluble stabilizers to maintain their enzymes and proteins.

Koffler (1957) has proposed that the uniqueness of true thermophiles lies in the relative stability of their critical molecules or structures. He has shown that flagella of thermophiles are more resistant to urea and acetamide than are mesophile flagella. Inasmuch as urea and acetamide are regarded as compounds capable of breaking hydrogen bonds, it seems likely that more 'effective' hydrogen bonding is involved in the relatively thermostable flagella of thermophiles. Similar studies conducted by Friedman (1961) and Fry (1961) seem to substantiate these findings.

Several studies involving factors capable of inducing mesophilic bacteria to grow at thermophilic temperatures have been reported. Sie et al. (1961a, b) have observed that cells of B. stearothermophilus contain a principle which when added to fresh culture medium permits an inoculum of a mesophilic organism to grow at 55C. Biochemical properties of the factor suggest that it is nucleoprotein in nature. This induced thermophily was carried from one transfer to another for an indefinite period when yeast autolysate was also provided in the medium. Oates et l. (1961) reported the presence of a thermophily factor in cell-free culture filtrates of a cellulose digesting mesophile-thermophile

mixed culture grown at 65C. When a mesophile was inoculated into the sterile filtrate, undiluted or at 50% concentration, growth of the mesophile at 65C was excellent; but, on inoculation in a 10% concentration of the filtrate, the mesophile demonstrated only moderate growth at 65C.

Studies involving various physiological properties of mixed bacterial cultures have proven useful from an applied biological viewpoint. Carbohydrate fermentation phenomena were investigated by Castellani (1926, 1927) in which several combinations of microorganisms in mixed culture produced fermentation patterns which were not characteristic of either organism in pure culture. By mixing various cultures and inoculating into carbohydrate broths, he could determine unknown carbohydrates present in the broths. Thus, symbiotic fermentation patterns may be used in the identification of carbon compounds.

Degradation of simple carbohydrates by symbiotic bacterial cultures can be paralleled with studies involving a more complex structure such as cellulose. A formidable amount of information has been collected pertaining to cellulose digestion by microorganisms--another specific form of carbohydrate fermentation. The mechanism of the symbiotic

utilization of cellulose by bacteria in termites and cattle has been reviewed by Hungate (1946). The evidence indicates that in both these forms the microorganisms ferment the digested cellulose and accumulate fermentation products which are absorbed and oxidized by the host. A classic model for the scheme of cellulose degradation by microorganisms was proposed by Reese and Levinson (1952):

cellulose $\xrightarrow[Cx]{}$ modified cellulose

modified cellulose $\xrightarrow{\text{hydrolytic enzyme}}$ cellobiose

cellobiose $\xrightarrow{\beta\text{-glucosidase}}$ glucose

According to Siu and Reese (1953) the microbe, in attacking cellulose, produces hydrolytic enzymes which are liberated into the medium. The insoluble substratum is hydrolyzed into soluble substances which diffuse into the cell where they are further metabolized.

According to Hungate (1950) the anaerobic cellulolytic bacteria which have been isolated fall into five categories: actinomycetes, thermophilic sporeformers, non-sporeforming rods and cocci, and mesophilic sporeformers. Most strains produce molecular hydrogen, carbon dioxide, ethanol, formic acid, acetic acid, lactic acid and succinic acid in varying proportions.

Cota (1954) studied aerobic mesophilic cellulose-decomposing bacteria which included Pseudomonas, Vibrio, and Sporocytophaga. He found that the presence of carbon dioxide stimulated the rate and pattern of the digestive reaction. Carbon dioxide was assimilated with the formation of key intermediates in the metabolism which altered the normal reaction pattern. Ellis (1955) found that glucose and cellobiose were the major end-products of cellulose digestion when he studied Sporocytophaga, Cellvibrio, and Corynebacterium. He did not determine the qualitative or quantitative distribution of organic acids formed in these degradations.

Thermophilic bacilli have been considered the most capable of causing cellulose digestion. Gyllenberg (1951) has commented on the descriptions of thermophilic clostridia capable of cellulose digestion which he feels, if they were investigated more thoroughly, would be found to be mixed with known mesophilic species. These findings suggest the possibility of closely associated symbionts causing the actual cellulose digestion. Enebo (1951) described three organisms capable of cellulose digestion only when they were symbiotically associated. These organisms were

Clostridium thermocellulaseum, Cl. thermobutyricum, and B. thermolacticus. All three fermented sugars to the same end-products when cultured individually. Another symbiotic cellulose digesting culture was studied by Oda and Ito (1950). The culture was capable of digesting 52% of the supplied cellulose with the production of cellobiose and glucose as intermediate decomposition products, and ethanol, acetaldehyde, acetic acid, carbon dioxide and hydrogen as final fermentation products.

Rumen microorganisms have been studied by Belasco (1956) and El-Shazly et al. (1961) for their production of volatile fatty acids as end-products of cellulose digestion. By changing the nature of carbohydrate substrate, they found different percentages of volatile fatty acids being produced.

Utilizing anaerobic mesophiles and thermophiles instead of rumen microorganisms, Isotalo (1951) and Hajny et al. (1951) reported the accumulation of acetic and lactic acids as principle end-products of carbohydrate fermentation by normal cellulose digesting bacteria isolated from bogs.

In general, when cellulose is attacked by microorganisms, common end-products detected are glucose and cellobiose (Enebo, 1949; Reese et al., 1952). In actively

growing cultures the further metabolism of cellobiose occurs within the organism. Too much cellobiose is known to be inhibitory to the hydrolysis of cellulose, therefore, removal of this compound by the digesting microorganism is advantageous. When Alexander (1961) studied the characteristics of cellobiose phosphorylase from cell-free extracts of Cl. thermocellum he found that this enzyme catalyzed the phosphorolysis of cellobiose with the formation of equimolar quantities of glucose and glucose-1-phosphate. The reaction is reversible, for equimolar quantities of cellobiose and inorganic phosphate are formed from glucose and glucose-1-phosphate.

Levinson et al. (1951) studied the products of enzymatic hydrolysis of cellulose and its derivatives. They detected cellobiose, in hydrolyzates, earlier than glucose. The cellobiose was rapidly converted to glucose when additional β-glucosidase was added to the reaction mixture. These observations support the idea of the direct utilization of cellobiose by the organism, a fact which had not seemed evident in their earlier studies.

Since cellulase has been shown to be a constitutive enzyme of Cl. thermocellum (Hammerstrom et al., 1955), the

production of an extracellular cellulase is no longer a controversial matter. Ke (1964) utilized a mixed mesophile-thermophile culture derived from Cl. thermocellum in the study of bacterial degradation of cellulose at 65C. Earlier studies of cellulose digestion by bacteria (Levinson et al., 1951) indicated that the enzyme capable of hydrolyzing a straight chain of anhydro-glucose units in β-1,4-glucosidic linkage acted upon the chain to produce glucose directly without a cellobiose intermediate. In the study completed recently by Ke (1964) the sugars which accumulated after cellulose digestion were found to be glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose. Since cellotriose and cellotetraose were present in relatively high concentrations, he proposed that they should be considered as intermediates of cellulose degradation rather than products of transglucosidation. The formation of these oligosaccharides is an indication that the cellulase system of his mesophile-thermophile mixed culture must cleave the cellulose chain at random rather than in an end-wise fashion.

Cl. thermocellum (Viljoen et al., 1926) was originally described as a pure culture thermophile which digested cellulose at 65C. McBee (1950, 1954) tested many anaerobic

thermophilic cellulolytic bacteria and concluded that all these strains should be considered as Cl. thermocellum. He claimed that the original culture of Viljoen was a mixed culture from which McBee later isolated a pure culture thermophile. Quinn and Tetrault (1950) also isolated a pure culture thermophile capable of cellulose digestion from an original Cl. thermocellum culture of Viljoen. Quinn (1949) reported that the thermophile was microaerophilic, motile, with an optimum temperature for cellulose digestion between 62-65C. Oates et al. (1963) studied the biochemical characteristics of the mesophilic-thermophilic organisms isolated from Quinn's thermophilic culture. These mixed culture symbionts demonstrated vigorous cellulose digestion which was not evident when either was in pure culture. Cell-free filtrates of the symbiotic culture protected the mesophile against thermophilic temperatures which are otherwise lethal to it. The mesophile essentially retains the same biochemical patterns following incubation at 65C in filtrates of the mixed culture as it showed with serial passage at 30C. Ecological studies of the pure and mixed cultures of this mesophile and thermophile show complex interrelationships in metabolic patterns. When both organisms are growing together

at thermophilic temperatures, the composite metabolic pattern is markedly different than that of either the mesophile or the thermophile.

MacLeod and Murray (1956) reported that cellulose digestion by rumen microorganisms was markedly stimulated by fishery by-products such as whale and herring solubles. Other factors present in yeast extract were found to be an absolute requirement for cellulose digestion (Quinn, 1949). The active ingredients in yeast extract capable of supporting cellulose digestion by Cl. thermocellum were found to be heat-stable and resistant to 1 N NaOH and 1 N HCl. The essential factor(s) were adsorbed on Norite at pH 3 or Clarite at pH 6-7, but not by Filter-Cel at pH 7, nor Norite at pH 6.6, 8, or 10. The factor(s) could not be replaced by B vitamins, amino acids, or nuclides. Chemical and biological similarities of the yeast extract factor(s) to kinetin, as described by Miller et al. (1956), suggested their possible identity.

The history of kinetin is relatively complex beginning with the isolation of the wound hormone, traumatin (English and Bonner, 1937; English, et al., 1939). This was one of the first hormones reported which elicited renewed growth

activity in the parenchymatous cells of the bean mesocarp.

Caplin and Stewart (1948) studied the effect of coconut milk on the growth of explants from carrot root. The active principle was not a constituent of the ash, nor was it any of the following vitamin-like growth factors: thiamine, niacin, pyridoxine, or glycine. The substance was stable to prolonged autoclaving and since its activity decreased on dialysis, they concluded that the compound must be a relatively small molecule. In related work, tobacco pith tissue also showed marked enlargement when water extracts of coconut milk or malt were added (Jablonski and Skoog, 1954).

Miller et al. (1956) isolated kinetin in pure form from heated deoxyribonucleic acid (DNA) preparations. They found that the compound would promote cell division in various plant tissue cultures in concentrations as low as 1 ug/L. Kinetin was shown by degradation and synthesis to be 6-furfuryl-aminopurine. Since Miller and his co-workers reported the isolation of kinetin, several groups have published data on the synthesis of similarly substituted adenine derivatives (Kissman and Weiss, 1956; Elion et al., 1952; Bullock et al., 1956; and Daly and Christenson, 1956).

Several studies have been conducted relative to the

effect of kinetin on microbial systems. Kennell (1959) found that the growth rates of Amoeba proteus, Escherichia coli, and two species of yeast were changed by the presence of kinetin and/or the plant auxin, indole acetic acid (IAA). The observed increase or decrease in the time between mitosis and daughter-cell growth may be ascribed to an action on the growth-regulating system which is duplicated at this stage of the cell cycle. Kennell postulated that ribonucleoprotein is the key component in this system, and there is evidence that kinetin may alter its metabolism.

Braun (1956) found that a breakdown product of bacterial DNA, obtained after exposure of DNA to DNase, will kill relatively avirulent cells without inhibiting the propagation of virulent cells. Thus, DNA plus DNase added to initially non-S cultures of Brucella species will promote the rapid establishment of spontaneously arising virulent S mutants. Actually, this selective activity is not associated with the initial breakdown product of DNA that is obtained after exposure to DNase, but, as determined with the help of filtrates from supplemented S cultures, the active component results from the action of S cells upon this initial DNA breakdown product. Therefore, avirulent non-S cells are not

inhibited by DNA plus DNase until a few S cells occur spontaneously in the growing population, or until a filtrate from S cultures exposed to DNA plus DNase is added. Braun has found that the active DNA breakdown product is heat-stable and alcohol precipitable. Its activity could not be duplicated by any of the commercially available purines, pyrimidines, nucleotides, or nucleosides that he tested. When he tested kinetin in this system, he found that it had a selective activity similar to that obtained after addition of DNA plus DNase to Brucella or pneumococcus cultures, and it also inhibited avirulent cells without affecting virulent cells, thus promoting population changes from non-S to S. He is in the process of determining whether the selectively active product from bacterial DNA is identical with or similar to kinetin, and whether such selective substances play any significant role in supporting the establishment of virulent cell types in vivo.

Lansford et al. (1958) have developed an assay for kinetin by means of the augmented inhibitory effect exerted by this compound upon growth of pteridine-inhibited Lactobacillus arabinosus in the absence of thymidine. Zones of inhibition were used as response indicators.

Maruzzella and Garner (1963) studied the effect of kinetin on the growth of animal and plant pathogenic bacteria. The test organisms were B. megaterium, E. coli, Staphylococcus aureus, Corynebacterium michiganense, Erwinia carotovora, and Agrobacterium tumefaciens. Concentrations of kinetin from 10^{-5} to 10^{-10} molar were incorporated into nutrient broth media, autoclaved, and inoculated with the test organisms. After 24 hr incubation, plate counts were made and the number of organisms per ml were compared to controls. The results showed that within certain concentrations, 10^{-7} to 10^{-9}, kinetin exerted a marked stimulatory effect on B. megaterium and A. tumefaciens, and to a lesser degree on E. coli, S. aureus, and E. carotovora.

Quinn et al. (1963) reported the support of cellulose digestion by Cl. thermocellum in a kinetin-supplemented basal medium. Strain C & E of Cl. thermocellum, a mixed mesophile-thermophile culture which symbiotically digests cellulose at 65C in a yeast extract medium, has now been shown to digest cellulose with typical yellow chromogenesis in a basal salts medium supplemented with 1 ug/L of kinetin.

The mode of action of kinetin has not been discovered as yet, but several workers have postulated mechanistic

theories to support observed phenomena. In a review by Miller (1961) purines are discussed as being implicated in all phases of plant growth. These compounds are structural parts of several of the energy handling systems, unit transfer systems, and the genetic communication machinery. Kinetin has and will serve as a biochemical compound which may help explain the chemical basis of growth. Skoog and Miller (1957) stated that the exact chemical nature of the interaction between kinetin and cells remains to be determined, but there can be little doubt that it is involved in nucleic acid metabolism including nucleic acid synthesis. It is possible to visualize, therefore, an essentially nuclear mechanism of growth regulation which possesses recognized means for interaction with the cytoplasm and which also is considered to be represented rather directly in the synthesis of large molecular structural units of the cell walls.

In order to check whether kinetin actually does effect protein synthesis directly at the stage of the formation of the protein molecule, or whether its action is directed only toward an enrichment of the tissues by protein precursors, Kulayeva and Vorob'yeva (1962) tested the effect of kinetin

in the presence of chloramphenicol, a specific inhibitor of protein synthesis. According to the presently existing notions, chloramphenicol does not depress the formation of amino acids, their primary activity, or the synthesis of the peptide bond. Its action is manifested directly at the stage of the formation of ribonucleic acid (RNA) or an anomalous structure in the cell. Therefore, chloramphenicol should prevent the positive effect on protein synthesis of kinetin or of any other substance whose action is directed toward the earlier stages of this process. In their study involving this effect in barley leaves, Kulayeva and Vorob'yeva found that the leaves treated with both chloramphenical and kinetin contained twice the amount of protein as the leaves treated with just chloramphenicol. Therefore, the action of kinetin on protein synthesis in leaves was not suppressed by chloramphenicol. These workers concluded that the action of kinetin on protein synthesis, just as the action of chloramphenicol, is intimately associated with the processes occurring directly in the formative stage of the protein molecule. The mechanism of this action will undoubtedly be solved in the future.

MATERIALS AND METHODS

Taxonomic Identification of the Symbionts

Preparation of culture inocula

Ten bacterial cultures were screened through routine identification tests. These included four mesophiles: #33 and #57, with acquisition numbers which refer to strains isolated from the original mixed culture of Viljoen *et al.* (1926); *B. megaterium* ATCC 7954, and *B. megaterium* ATCC 8245; two mixed mesophile-thermophile cultures, namely NTM, and C & E; and four pure-culture thermophiles: TNTM, and TC & E, which are strains isolated from the original mixed culture; *B. stearothermophilus* ATCC 7953, and *B. stearothermophilus* ATCC 7954. Each of the ATCC cultures was used as a reference for identification of the unknown strains. Stock cultures were maintained on nutrient agar slants. The source of inocula for duplicate observations of each specific test consisted of 24 hr nutrient broth cultures, which contained from 10^5 to 10^6 viable cells per 0.1 ml. The test organisms were grown in 10 ml volumes of nutrient broth in dropper bottles equipped with droppers calibrated to deliver 0.1 ml per drop. Results were read at appropriate time intervals,

depending upon the nature of the test, and all positive cultures were sub-cultured in duplicate by loop, and incubated at 30C or 65C, as required by the test organism.

Arrangement of culture tubes for inoculation

Four aluminum culture tube racks containing 360 holes each were purchased from the H. M. Chemical Co., Ltd., Los Angeles, Calif. Culture tubes, 13 X 120 mm, Arthur H. Thomas Co., Philadelphia, Pa., were arranged in the racks to provide for duplicate observations, and sub-cultures, of each test on each organism. Closures for the culture tubes were 13 mm aluminum caps. Two of these culture tube racks were used for the incubation of the mesophiles, and two similar racks were used for the incubation of the thermophiles.

Determinations employed

Approximately seventy diagnostic tests were conducted on the ten cultures under study. Media utilized in these tests were the same as those recommended for identification of this group of organisms by Conn and Pelczar (1957), and by Lord (1959). The specific tests employed are tabulated in the RESULTS section.

Evidence for the Presence of a Thermophily Factor

Auxanographic plates

D58 medium (Quinn, 1949) which contains cellulose as the principle carbon source, was solidified by the addition of 1.5% agar, for plating. A 3-4 day old C & E culture, a mixed culture of mesophile-thermophile, was streak inoculated at one side of an agar plate. Three to five streaks of a 24 hr mesophilic culture, #33, were made at right angles to the first streak. After 12-24 hr incubation at 65C, colonies of the mesophilic organism appeared adjacent to the line of the perpendicular feeder streak. No such growth occurred on control plates which lacked the C & E culture feeder streak. In an attempt to extend the growth response to greater distances from the feeder streak, the plates were refrigerated for 24 hr to allow time for more complete diffusion of the apparently diffusible factor, following this holding period the plates were incubated at 65C. There seemed to be no difference in growth response of the mesophile, whether the plates were refrigerated overnight, or not, prior to incubation at 65C. This seemed to indicate that the factor had a rapid rate of diffusion through agar at room temperatures, but did not reach effective concentration at points remote

from the feeder streak.

Cell-free filtrates

C & E cultures which had been grown at 65C for approximately 5 days were centrifuged at 37,000 X G for 45 min in a Servall centrifuge, Model SS3, to remove viscous products of cellulose fermentation. The resulting 'supernatant' was decanted and passed through a Millipore filter of 0.30 μ porosity to effectively sterilize the cell-free filtrate. When 0.1 ml of a 24 hr old pure culture mesophile was inoculated into this sterile filtrate, employed undiluted or at a 50% concentration in D58 medium, growth of the mesophile, #33, at 65C was excellent; but, at 10% concentration of the filtrate in D58 medium, the mesophile demonstrated only moderate growth at 65C. In initial experiments, un-inoculated control filtrates incubated at 65C remained sterile and free of precipitate.

Inactivation of the thermophily factor

Difficulties in assaying the 'thermophily factor' content of cell-free filtrates were encountered when it was found that un-inoculated control filtrates formed a white, flocculent precipitate when incubated for 2 hr at 65C or when heated to boiling for less than 1 min. When cell-free

culture filtrates were treated in this manner, they lost their capacity for protecting mesophiles at thermophilic temperatures. Examination of the white floc-precipitate from sterile D58 medium, under two different electron microscopes, RCA EMU 2A and 50KV EMU 3, in the Biophysics Department, Iowa State University, Ames, Ia., showed small coccoid particles connected by fine strands of filamentous material. Magnifications of the micrographs ranged from 1,400X to 37,000X.

Amino acid analysis of flocculent precipitate*

Eight-hundred ml of C & E culture filtrate were obtained as previously described. The white floc-precipitate was prepared by heating the filtrate to boiling and allowing the floc to settle. The resultant precipitate was washed three times in distilled water, placed in a drying dish, and allowed to dry at 65C. The total floc yield was 500 mg. Of this total, 100 mg were hydrolyzed in 10 ml of 6N HCl for 24 hr in an autoclave at 15 lb steam pressure. The hydrolyzed sample was centrifuged at 5,000 rpm for 15 min to remove humin material. Five ml of the sample were de-proteinized

*Determined by Al Clark, Swine Nutrition Department, Iowa State University, Ames, Iowa. 1962.

with a 1% solution of picric acid; a heavy precipitate formed with the addition of 25 ml of the picric acid solution. Following de-proteinization, the filtered sample was passed through a Dowex 2 column to remove the picric acid, and the resultant solution was reconcentrated by evaporation to a volume of 10 ml, then the solution was diluted with 0.02N HCl up to a volume of 10 ml. A 1 ml sample of this solution was analyzed on a Technicon Autoanalyzer for amino acid composition.

Since the acid hydrolysis of the floc-precipitate was incomplete, the amino acid determination was at best only qualitative, and included cysteic acid, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and histidine. Micro-Kjeldahl determinations on 100 mg of dried floc-precipitate showed 10% nitrogen present.

Media Preparation

Media containing yeast extract and/or chemical compounds substituted for yeast extract

Media were formulated which contained compounds substituted for yeast extract, in an attempt to rid sterile

cell-free filtrates of the precipitate which had been forming at 65C. This was done since it was felt that unknown ingredients in yeast extract (Difco) may have been causing the formation of the white, flocculent precipitate.

D58 medium was prepared with Basamin-Busch yeast autolysate, Anheuser-Busch, St. Louis, Mo., substituted in equal amount for the regular Difco yeast extract ingredient in the medium. Other media employed in this phase of the study were formulated as shown in Table 1.

The vitamin ingredients were added from stock solutions of each vitamin, and the pooled vitamin solution was brought up to a volume of 1 liter. Sterilization of this solution was accomplished by filtering through a GS type Millipore filter of 0.22 μ porosity. Two ml of the composite vitamin solution were added to each 100 ml of Defined Medium contained in each culture flask. After the Defined Medium and the Vitamin-Free Medium were prepared, 1 ml of the C & E stock culture was added to each of the test flasks, which were then incubated at 65C for 5 days. Both of these media supported growth of the C & E culture, including cellulose digestion. It was concluded that the Vitamin-Free Medium seemed to be adequate for cultivation of the symbionts as

Table 1. Ingredients employed in test media*

Vitamin-Free Medium		Defined Medium**	
Ingredient	Amt/L	Ingredient	Amt/L
$(NH_4)_2SO_4$	1.3 g	Thiamine	0.225 mg
$CaCl_2$	0.13 g	Riboflavin	0.225 mg
$MgCl_2 \cdot 6H_2O$	2.6 g	Niacin	2.25 mg
KH_2PO_4	1.43 g	Ca-Pantothenate	0.45 mg
$K_2HPO_4 \cdot 3H_2O$	7.2 g	Pyridoxine	0.112 mg
L-arginine	49.1 mg	Biotin	0.014 mg
L-histidine	21.3 mg	Folic acid	0.09 mg
L-lysine	91.4 mg	Choline	9.00 mg
L-tyrosine	56.2 mg		
L-tryptophan	14.0 mg		
L-phenylalanine	49.1 mg		
L-cystine	22.5 mg		
L-methionine	28.1 mg		
L-threonine	46.4 mg		
L-leucine	90.0 mg		
L-isoleucine	66.0 mg		
L-valine	67.5 mg		
Uracil	20.0 mg		
Guanine · HCl	20.0 mg		
Adenine · HCl	20.0 mg		
Cellulose (Avicel)	13.0 g		

*Amino acid and vitamin levels are equivalent to Basamin-Busch yeast autolysate analysis.

**Also add the same ingredients as shown in Vitamin-Free Medium.

serial passage of the mixed culture in this medium supported cellulose digestion for at least 6 successive transfers, when transfers were made at 5 day intervals.

In an attempt to simplify the ingredients of the Vitamin-Free Medium, Erlenmeyer flask cultures containing 100 ml of Vitamin-Free Medium were compared with similar flask cultures containing, on the one hand, the same medium minus the growth factors, adenine, guanine, and uracil; and, on the other hand, Vitamin-Free Medium minus the amino acid components of this medium. The three media were inoculated with 1 ml of the C & E culture and were incubated at 65C for 5 days. Only the complete formulation of Vitamin-Free Medium supported growth of the symbionts and cellulose digestion; however, sterile filtrates from this medium, after growth of the C & E mixed culture, continued to allow the formation of the white, flocculent precipitate when incubated at 65C, whereas un-inoculated Vitamin-Free Medium control tubes did not form the white, flocculent precipitate. This seemed to indicate that materials associated with cellulose digestion were responsible for floc-precipitate formation.

Since it was felt that high concentrations of free

phosphate ion seemed to favor the production of floc-precipitate forming materials in the Vitamin-Free Medium, an alternative medium was formulated which was designated Oates' Defined Medium (ODM). In ODM, sodium-beta-glycerolphosphate was substituted for KH_2PO_4 and K_2HPO_4 of the Vitamin-Free Medium. A complete list of ODM ingredients is shown in Table 2.

Complete ODM, minus cellulose, was filter sterilized through an HA type Millipore filter, 0.45 μ porosity, to avoid denaturation of the vitamin ingredients, and also to avoid the precipitation which occurs upon autoclaving of the medium. One-hundred ml quantities of sterile ODM were pipetted into sterile 250 ml Erlenmeyer flasks containing 1.3 g of Avicel cellulose (American Viscose Corp., Marcus Hook, Pa.). One ml each of 24 hr D58 cultures of #33, NTM, C & E, TNTM, and TC & E, was added to separate culture flasks of ODM which were incubated at 30C, in the case of #33, and at 65C, in the case of the thermophiles and mixed cultures. All of the test cultures grew well in ODM, but cellulose digestion was not evident in the culture flasks inoculated with the normally cellulolytic mixed cultures, NTM and C & E.

Table 2. Ingredients of Oates' Defined Medium (ODM)*

Ingredient	Amt/L
Glycerol	40.0 ml
DL-asparagine	4.0 g
Sodium-beta-glycerolphosphate	6.0 g
KCl	100.0 mg
$MgSO_4 \cdot 7H_2O$	800.0 mg
$CaCl_2$	50.0 mg
L-histidine	50.0 mg
L-methionine	60.0 mg
L-tryptophan	60.0 mg
Thiamine	150.0 ug
Riboflavin	150.0 ug
Nicotinic acid	1600.0 ug
Ca-Pantothenate	1000.0 ug
Pyridoxal	75.0 ug
Biotin	9.0 ug
Folic acid	60.0 ug
Cellulose (Avicel)	13.0 g

*pH adjusted to 7.0 prior to sterilization.

Since ODM would not support cellulose digestion, a simple Basal Medium (Table 3) was formulated to aid in the synthesis of a minimal medium with the necessary metabolites for cellulose digestion. Two 100 ml batches of the Basal Medium were prepared. One batch was filter-sterilized through an HA type Millipore filter and designated (FBM), for Filtered Basal Medium. The other batch (ABM), Autoclaved

Table 3. Basal Medium for cultivation of test organisms*

Ingredient	Amt/L
Maltose	5.0 g
Sodium-beta-glycerolphosphate	6.0 g
$MgSO_4 \cdot 7H_2O$	0.8 g
$(NH_4)_2SO_4$	1.3 g
$CaCl_2$ (anhydrous)	50.0 mg

*pH should be adjusted to 7.0 prior to sterilization.

Basal Medium, would be inoculated and incubated in the same way as FBM to test the effect of the method of sterilization on the nutritional properties of this medium.

Five ml volumes of each medium were added separately to sterile 16 X 125 mm Kimax screw-cap culture tubes, each containing 0.05 g of Avicel cellulose. 0.1 ml of each of the test cultures, mesophiles, thermophiles, and mixed cultures, was added to duplicate tubes of each medium. All tubes were incubated for 5 days at the optimum growth temperature for each organism.

FBM seemed to be more nearly optimal for growth of the test organisms than did ABM; however, neither medium supported cellulose digestion by the mixed cultures, NTM and

C & E. Further research on the nutritional requirements for cellulose digestion is presented in the next section.

Kinetin-supplemented basal medium

Quinn et al. (1963) found that kinetin, when added to a basal salts medium, could be substituted for active ingredients in yeast extract which support cellulose digestion by the C & E mixed culture at 65C. By deleting maltose and $MgSO_4$ from the Basal Medium (Table 3) and adding, per liter, 1 μg of kinetin, 1.43 g of KH_2PO_4, 7.2 g of K_2HPO_4, and 2.6 g of $MgCl_2 \cdot 6H_2O$, the kinetin-supplemented basal medium (Table 8) was formulated.

In Vitro Studies on the Thermophily Factor

Parabiotic chambers

In an attempt to circumvent the problems associated with filtration, spontaneous floc formation, and 65C temperature denaturation of the thermophily factor, 'parabiosis' was attempted; for this study parabiotic chambers were obtained from the Bellco Glass Co., Vineland, N. J. It was hoped that with the aid of this apparatus, it would be possible to study the in vitro production of the thermophily factor by the mixed C & E cultures growing at

65C.

The parabiotic chamber consists of a pair of open glass tubes which fit together at plane-ground surfaces. A spring holder holds the 'right' and 'left' arms of the parabiotic chamber in place. A Millipore filter can be inserted between each pair of arms, thus creating a culture vessel in which the flow of solutes, but not cells, may occur randomly from one arm to the other. The porosity of the Millipore filter employed is, of course, the limiting factor relative to the size of a molecule that can pass from one arm to its apposed partner.

In this study, three parabiotic chambers were used in a common holder. Prior to sterilization of the parabiotic chamber, one HA type Millipore filter of porosity 0.45 μ was placed between each of the three pairs of arms. Screw clamps were used to insure a firm fit between the sets of apposed arms, and rubber stoppers were inserted in the open end of each parabiotic arm. The entire apparatus was autoclaved for 25 min at 121C.

Following sterilization, 12 ml of a 48 hr mixed C & E culture grown at 65C in D58 medium were pipetted aseptically into three left-arms of the parabiotic chambers. Twelve ml

of sterile supernatant from D58 flasks were pipetted into each right arm of a set. 0.2 ml of a 24 hr mesophilic culture, #33, grown at 30C in D58 medium was aseptically added to two right arms apposed to those left arms containing the C & E mixed culture. The remaining parabiotic chamber served as an un-inoculated control. The parabiotic chambers were then incubated at 65C and were observed at appropriate time intervals. Under these conditions the mesophile, #33, on occasion, survived the 65C incubation for a period of time ranging from 24-80 hr, as determined by subculture in D58 medium and incubation at 30C and 65C; however, further work in this area must be completed before positive conclusions can be drawn.

Evidence for the Presence in C & E Culture Filtrates of a Growth Factor for the Mesophiles

Effect of addition of sterile filtrates to carbohydrate fermentation test-media

Four carbohydrates; cellobiose, cellulose, glucose, and trehalose, were used as energy sources in an experiment designed to characterize a stimulatory response found upon adding sterile filtrate, from a 72 hr mixed C & E culture

grown at 65C, to fermentation media inoculated separately with two mesophilic isolates, #33 and #57. These twelve different media which were dispensed in culture tubes in 5 ml amounts included: (1) four media containing, individually, 0.5% of the four indicated carbohydrates plus sterile filtrate diluted in each medium to a final concentration of 10%, (2) four media containing the same concentration of each carbohydrate, individually, plus filtrate as described in (1) above, but including 'synthetic base' as specified by Conn and Pelczar (1957), and (3) four media containing 0.5% of the four carbohydrates, individually, plus synthetic base.

When C & E culture filtrate was added, a stimulatory effect was noted as indicated by increases in the amount of acid formed during carbohydrate fermentation by #33; therefore, the experiment was repeated and enlarged to also test filtrates of sterile D58 medium for the same stimulatory effect.

In this second experiment, twenty media were prepared employing five different media for each carbohydrate under study. The treatments tested were cellulose, cellobiose, glucose, and 'sugar-free', which served as a control. A

complete list of media used is shown in Table 4.

Table 4. Media utilized in detecting the stimulatory growth factor*

Synthetic Base	Syn Base plus 10% D58 Filt	Syn Base plus 50% D58 Filt	Syn Base plus 10% C&E Filt	Syn Base plus 50% C&E Filt
None	None	None	None	None
Cellulose	Cellulose	Cellulose	Cellulose	Cellulose
Cellobiose	Cellobiose	Cellobiose	Cellobiose	Cellobiose
Glucose	Glucose	Glucose	Glucose	Glucose

*Concentration of carbohydrates was 0.5%. All media were adjusted to pH 7.0 prior to sterilization. Add 1 ml/50 ml media of 0.1% brom thymol blue in 50% ethanol, as pH indicator for titration.

The media were dispensed in 5 ml amounts and were inoculated, as previously described, with the mesophilic strains, #33 and #57. Each fermentation test was titrated in duplicate for each organism with duplicate un-inoculated controls for each test. The incubation period was 5 days at 30C.

The addition of sterile C & E culture filtrate to the

glucose and cellobiose media at 50% (v/v) concentration had a very significant effect on the amount of titrable acidity produced by fermentation reactions of culture #33. Therefore, the same filtrate at the same level was tested further by comparing glucose, xylose, and trehalose as carbohydrate energy sources.

Effect of addition of mixed culture filtrate to carbohydrate media

Carbohydrate fermentation media (Table 5) were combined with sterile mixed C & E culture filtrate in 50% (v/v) concentration. The purpose of this experiment was to determine differences between the abilities of various carbohydrates to support the observed stimulatory effects of C & E filtrates on fermentation. Media were dispensed in 5 ml amounts, and included, for each medium, 4 culture tubes for inoculation with #33 and 2 culture tubes to serve as controls.

Results of this experiment indicated that glucose was superior to xylose and trehalose as carbohydrate energy sources in demonstrating the stimulatory effect of 50% (v/v) sterile C & E culture filtrate on the fermentation pattern of culture #33. Therefore, glucose was selected as the test carbohydrate to be utilized in future studies

designed to assess differences in the stimulatory capacity of sterile filtrates from several different cultural sources.

Table 5. Media utilized in assessing differences between carbohydrates in the ability to support the fermentation stimulating effect of mixed C & E culture filtrate*

Control	Glucose 0.5%	Xylose 0.5%	Trehalose 0.5%
Syn base 50% filtrate	Syn base 50% filtrate	Syn base 50% filtrate	Syn base 50% filtrate

*All media were adjusted to pH 7.0 prior to sterilization. Add 1 ml/50 ml of media of 0.1% brom thymol blue in 50% ethanol, as pH indicator for titration.

Tests for stimulatory effect of filtrates from various cultural sources

Sterile filtrates, prepared as previously described, were obtained from the following sources: (1) D58 medium; (2) 3-day-old pure culture mesophile, #33, grown in D58 medium at 30C; (3) 3-day-old mixed C & E culture grown in D58 medium at 65C; and (4) 3-day-old pure culture thermophile, TC & E,

grown in D58 medium at 65C.

Prior to the addition of 50% filtrate (v/v) to 0.5% glucose media (Table 6), each filtrate was tested for the presence of glucose by dipping a 1 1/2 inch strip of Tes-Tape, Eli Lilly and Co., Indianapolis, Ind., into the respective filtrates. Strips were allowed to develop for 1 min at 25C. Percentage of glucose present was read by comparing the darkest area on the test strip with the color chart on the Tes-Tape dispenser. The results indicated that the filtrates were glucose-free, thus eliminating glucose as the 'growth factor'.

Procedures utilized for dispensation and sterilization of the media were as described previously.

Of the sterile D58 medium culture filtrates tested, C & E culture filtrate seemed to contain the optimum (or maximum) level of growth factor required to demonstrate the stimulatory fermentation response of culture #33. Consequently, sterile C & E culture filtrate from D58 medium was selected as a standard to be used in future studies to determine the relative amounts of growth factor that might be present in other culture filtrates.

Table 6. Ingredients of the four 0.5% glucose media employed with 50% (v/v) sterile culture filtrates*

Filtrate Source			
D58	#33	C & E	TC & E
Syn base	Syn base	Syn base	Syn base
0.5% glucose	0.5% glucose	0.5% glucose	0.5% glucose

*All media were adjusted to pH 7.0 prior to sterilization. Add 1 ml/50 ml of media of 0.1% brom thymol blue in 50% ethanol, as pH indicator for titration.

The Effect of Kinetin on Fermentation Patterns

Procedure for the addition of kinetin to carbohydrate media

Since kinetin had been shown to stimulate division of cells (Strong, 1958), it was felt that this compound should be tested for its ability to substitute for D58 culture filtrates, which may contain kinetin, in stimulating #33 to produce increased amounts of titrable acidity when inoculated into carbohydrate media. A kinetin stock solution was prepared by dissolving 5 mg of kinetin in 100 ml of acidified distilled water. Eight fermentation media (30 ml each) were prepared by adding kinetin in the following final

concentrations (μg/ml) to the 0.5% glucose, synthetic base, fermentation medium: 0.000, 0.0005, 0.001, 0.002, 0.005, 0.010, 0.250, and 1.000. All tubes were inoculated with #33 culture and were incubated as previously described.

Procedure for the addition of buffer to kinetin-carbohydrate media

Since D58 medium contains 1.43 g/L of KH_2PO_4 and 7.2 g/L of K_2HPO_4 to act as a buffer system in the medium, it was felt that an equivalent amount of buffer had to be incorporated into the synthetic base-kinetin-carbohydrate media to equalize the buffer capacity in the two systems. Two alternative buffer systems that were tested, but which proved to be unsatisfactory, were 0.2M tris buffer, and 0.4M sodium-beta-glycerolphosphate combined with 0.3M L-cysteine.

In preliminary cultural tests, it was found that optimal concentrations of buffer resulted from the use of 0.005M KH_2PO_4 and 0.016M K_2HPO_4 or 0.011M KH_2PO_4 and 0.032M K_2HPO_4. The latter concentration is identical to that employed in D58 medium.

Stock solutions employed for media preparation

Fermentation media for this experiment were prepared as shown in Table 7. Stock solutions of synthetic base, and

Table 7. Ingredients employed in the preparation of kinetin-carbohydrate media utilizing stock solutions of phosphate buffer, synthetic base, kinetin, and glucose*

Final Buffer Concentration	
1/2 X 0.005M KH_2PO_4 0.016M K_2HPO_4	X 0.011M KH_2PO_4 0.032M K_2HPO_4
2.5 ml Buffer stock (10X)	5.0 ml Buffer stock (10X)
5.0 ml Syn base stock (10X)	5.0 ml Syn base stock (10X)
5.0 ml Kinetin dilution	5.0 ml Kinetin dilution
32.5 ml Distilled water	30.0 ml Distilled water

*Add 5 ml of sterile 5% glucose stock solution following media sterilization. Add 1 ml/50 ml total media of 0.1% brom thymol blue in 50% ethanol prior to sterilization.

phosphate buffer, were made up at ten times their normal concentrations. A 5% glucose stock solution was autoclaved separately and added aseptically to each of the fermentation media. A kinetin stock solution that contained 100 µg/ml kinetin was utilized to prepare serial two-fold dilutions of kinetin that ranged from 0.0005 µg/ml to 1.000 µg/ml. Dilutions were made in 16 X 125 mm test tubes by serially transferring 5 ml aliquots into 5 ml of distilled water, after having made the initial 1: 9 dilution of the concen-

trated stock solution. A 5 ml distilled water blank served as a zero level kinetin control. The 26 media required were prepared in individual 125 ml Erlenmeyer flasks, and media were then dispensed as previously described. After initial experimentation on buffer systems had been completed, it was found, through statistical analysis, that a significant buffer by kinetin interaction did not exist; therefore, since buffer concentration was of no significant consequence in the expression of the kinetin effect, 1/2X buffer was arbitrarily selected as a standard buffer concentration to be employed in test media for future experimentation. Media were prepared as shown in Table 7 for 1/2X buffer concentration for the remainder of the experiments involving kinetin.

Preparation of inocula for kinetin-evaluation experiments

Both symbionts, cultures #33 and TC & E, were tested individually for a growth stimulatory effect by kinetin. Forty-eight hr nutrient broth dropper bottle cultures of the test organisms were utilized as inocula for this phase of the study. In the case of culture #33, the mesophilic isolate, 2 culture tubes per level of kinetin supplemented medium were inoculated, and 2 similar tubes served as uninoculated controls. Culture tubes were incubated at 30C

for 7 days. In the case of culture TC & E, the thermophilic isolate, 5 culture tubes per level of kinetin supplemented medium were inoculated and 2 culture tubes served as uninoculated controls. Culture tubes were incubated at 65C for 7 days. Each experiment was replicated twice to insure control of sampling and experimental variation.

Mode of Action of Kinetin on Bacterial Cells

In a nitrogen limited system

Two 250 ml nutrient broth cultures of #33 were grown at 30C for 24 hr. Both sets of cells were centrifuged aseptically at 5000 X G for 15 min, in metal centrifuge tubes equipped with metal lids. After the initial centrifugation, the supernatants were decanted and the cells were washed 3 times by centrifugation using 20 ml of sterile saline per tube per wash. Following this treatment, the cells were resuspended in 10 ml of sterile saline and were transferred to a sterile 125 ml Erlenmeyer flask. Each set of washed cells was serially diluted in sterile saline blanks for plating on nutrient agar plates at appropriate ten-fold dilution levels. The inocula, for later use, were standardized by this method and found to contain from 10^4 to 10^7 cells/ml.

Eighteen kinetin-supplemented media (containing kinetin from 0 to 1 μg/ml) with 1/2X buffer, as shown in Table 7, were prepared. Of these, 13 media had nitrogen deleted from the formulation of their synthetic base medium, whereas the other 5 media were prepared according to Table 7 and contained respectively, 0.000, 0.002, 0.016, 0.064, and 0.512 μg/ml of kinetin. These 5 media served as kinetin controls in assessing the effect of kinetin on cells grown in a medium in which nitrogen was not limiting, compared to its effect on cells grown in a nitrogen limited system. Five ml aliquots of the 13 media which were nitrogen limited were dispensed into sets of 6 culture tubes per medium; 2 tubes of each medium were inoculated with 0.1 ml of the first set of washed #33 cells, and 2 other culture tubes of each medium were inoculated in the same manner with the second set of washed #33 cells, while 2 culture tubes of each medium served as un-inoculated controls. Tubes of the other 5 media were inoculated as previously described.

In this experiment, the cells grown in the nitrogen limited media were found to be incapable of glucose metabolism. Arbitrarily, 3 of the 13 media were selected, those with 0.000, 0.016, and 0.512 μg/ml of kinetin, for

determining the number of viable cells present after 48 hr incubation at 30C; this was checked to assess the effect of kinetin on cell division. No increase in total viable cell counts over initial inocula was noted for cells grown in the nitrogen limited media. It was concluded from this that kinetin stimulated cell division of culture #33 in complete-nitrogen-containing-kinetin-media, but not in media which were nitrogen deficient. Kinetin does not seem to act as either an energy source or a fermentation co-factor in such systems, as the activity of the fermentative enzymes of culture #33 were not stimulated.

Demonstration of absence of a kinetin effect on cultural characteristics of culture #33

In an attempt to determine the possible effect of kinetin on the physiological characteristics of culture #33, eleven diagnostic tests were determined on cells grown for 24 hr at 30C in 15 nutrient broth dropper bottles containing from 0.000 to 5 μg/ml of kinetin. The diagnostic tests employed were: growth in nutrient broth at pH 9.6, in nutrient broth plus 5% NaCl, in Koser's citrate medium, and in anaerobic alkaline KNO_3; hydrolysis of starch agar, casein agar, and gelatin; production of nitrite from nitrate;

fermentation of glucose, but not of xylose or arabinose. All media were dispensed, inoculated and incubated as previously described. Tubes were read at 24 and 48 hr, depending on the test employed. It was found that there were no measurable differences in physiological characteristics notable at all the levels of kinetin that were tested. Therefore, it can be concluded that the cells are essentially not altered physiologically after incubation in the presence of kinetin.

The effect of kinetin on cell morphology

Culture #33 was grown in nutrient broth dropper bottle cultures and in glucose media containing kinetin (Table 7) at 0.000, 0.032, and 1.00 μg/ml; all culture tubes were incubated at 30C. Cells fixed with Bouin's for 5 min were examined on slides by phase microscopy with observations being made at 5 hr, 20 hr, and 6 days with respect to the nutrient broth cultures, and at 48 hr and 6 days with respect to the kinetin media. After 48 hr in the medium containing 1.00 μg/ml of kinetin, the cells have an abnormal arrangement which can best be described as being 'kinky'. In order to speculate with regard to this phenomenon, one would need more data to form a definite opinion.

Growth Stimulation Comparisons Between Kinetin and Yeast Extract as Supplements

Formulation of test media

Since filtrates from D58 medium were found to stimulate growth of culture #33, and since kinetin was found to act in a similar if not identical manner, media were formulated (Table 8) from which C & E mixed culture filtrates were obtained.

Sterile filtrates, as previously described, were obtained from the following sources: (a) 3-day-old C & E mixed cultures grown at 65C in media #'s 1 and 2, and (b) 10-day-old C & E mixed cultures grown at 65C in media #'s 3, 4, and 5. Since these latter media are not as nutritionally complete as media #'s 1 and 2, the longer incubation period was used to permit appreciable cellulose digestion to occur.

Addition of test media culture filtrates to fermentation media

After each of these 5 filtrates was obtained, they were used to supplement fermentation media (Table 9) which were prepared in 125 ml Erlenmeyer flasks.

All media were dispensed and inoculated as previously

Table 8. Ingredients incorporated in test media to compare the growth stimulations induced by kinetin and by yeast extract-sodium glycerophosphate (NGP)*

Ingredient (g/L)	Medium #1 D58	#2 D58 plus NGP	#3 D58 minus Yeast Extract	#4 Kinetin**	#5 Kinetin-Free
$(NH_4)_2SO_4$	1.3	1.3	1.3	1.3	1.3
Yeast Extract	4.5	4.5	-	-	-
$CaCl_2 \cdot 2H_2O$	0.13	0.13	0.13	0.13	0.13
$MgCl_2 \cdot 6H_2O$	2.6	2.6	2.6	2.6	2.6
KH_2PO_4	1.43	1.43	1.43	1.43	1.43
$K_2HPO_4 \cdot 3H_2O$	7.2	7.2	7.2	7.2	7.2
Sodium glycero-phosphate	-	6.0	-	6.0	6.0
Kinetin	-	-	-	10.0 µg	-
Cellulose (Avicel)	13.0	13.0	13.0	13.0	13.0

*Media adjusted to pH 7.15 with 2N NaOH prior to sterilization.

**Add kinetin (0.1 ml of 10 µg/ml stock) to each 100 ml of medium if to be used immediately. If the medium is to be stored, omit the kinetin at the time of preparation and add same just prior to use and re-autoclave the entire medium.

Table 9. Combinations of filtrates and fermentation media ingredients employed to determine differences in growth stimulation caused by kinetin and yeast extract*

Medium	Final Filtrate Concentration		
	0%	10%	50%
Control	3 ml 5% glucose 3 ml Syn base (10X) 24 ml Distilled water	-	-
#1	-	3 ml 5% glucose 3 ml Syn base (10X) 21 ml Distilled water 3 ml Filtrate #1	3 ml 5% glucose 3 ml Syn base (10X) 9 ml Distilled water 15 ml Filtrate #1
#2	-	3 ml 5% glucose 3 ml Syn base (10X) 21 ml Distilled water 3 ml Filtrate #2	3 ml 5% glucose 3 ml Syn base (10X) 9 ml Distilled water 15 ml Filtrate #2
#3	-	3 ml 5% glucose 3 ml Syn base (10X) 21 ml Distilled water 3 ml Filtrate #3	3 ml 5% glucose 3 ml Syn base (10X) 9 ml Distilled water 15 ml Filtrate #3

*Add 0.6 ml/30 ml of media of 0.1% brom thymol blue in 50% ethanol prior to sterilization, as pH indicator for titration. Add sterile filtrates aseptically following sterilization of remainder of the medium.

Table 9 (Continued)

Medium	Final Filtrate Concentration		
	0%	10%	50%
#4	-	3 ml 5% glucose 3 ml Syn base (10X) 21 ml Distilled water 3 ml Filtrate #4	3 ml 5% glucose 3 ml Syn base (10X) 9 ml Distilled water 15 ml Filtrate #4
#5	-	3 ml 5% glucose 3 ml Syn base (10X) 21 ml Distilled water 3 ml Filtrate #5	3 ml 5% glucose 3 ml Syn base (10X) 9 ml Distilled water 15 ml Filtrate #5

described. Five tubes of each medium were employed, 3 of which were inoculated with culture #33, and 2 of which served as un-inoculated controls. Tubes were incubated at 30C for 7 days.

Statistical Analysis of Fermentation Data

The fermentation experiments utilized throughout this study were designed as randomized blocks in some cases and factorial arrangements of treatments in other cases (Snedecor, 1956). The majority of the experimental designs involved two replications of an experiment to aid in the control of experimental and sampling variations.

The data were obtained by titrating the entire contents of each culture tube (plus 50 ml of distilled water to add volume) back to pH 7.0 with 0.001N NaOH, after the initial pH had been read on each individual tube with the aid of a Beckman pH Meter, Model H2, National Technical Labs., Pasadena, Calif. Control tubes were read first for each specific medium and were titrated to pH 7.0, if the pH reading had changed during incubation. Tubes which had been inoculated were read for pH, and if the reading was not the same as the control tube for that medium, the

contents were titrated back to pH 7.0, and the number of ml necessary to adjust to this pH minus the number of ml used for the control tube was recorded for statistical analysis.

RESULTS AND DISCUSSION

Taxonomic Identification of the Mixed Culture Symbionts

Mesophilic cultural characteristics, as evidenced in Tables 10 to 12, were utilized in the identification of the two unknown cultures, #33 and #57. Reference cultures B. megaterium ATCC 7051 and ATCC 8245 do not coincide physiologically with the unknown cultures on the following diagnostic tests (Table 10): growth in nutrient broth at pH 9.6; production of nitrite from nitrate; ability to sub-culture in Koser's citrate medium and urea medium; growth in anaerobic alkaline KNO_3 medium; growth at 10C in nutrient broth and in filtered D58 medium.

Carbohydrate fermentation data (Table 11) revealed cultural differences as evidenced by employing two basal media, non-synthetic and synthetic, in conjunction with 0.5% concentrations of various carbohydrates. Cultures #33 and #57 had identical fermentation patterns when grown in non-synthetic basal medium; however, they disagree on one characteristic, sucrose fermentation, when grown in synthetic basal medium. B. megaterium strains ATCC 7051, and ATCC 8245 do not ferment carbohydrates in non-synthetic basal medium;

Table 10. Initial transfer and sub-culture data (S) on mesophiles as determined by diagnostic tests observed after 48 hr incubation at 30C*

Test	Cultures							
	#33		#57		7051		8245	
	48	S48	48	S48	48	S48	48	S48
Growth in nutrient broth	+	+	+	+	+	+	+	+
at pH 9.6	+	+	+	+	-	-	-	-
with 2% salt	+	+	+	+	+	+	+	+
with 5% salt	+	+	+	+	+	+	-	-
KNO_3 reduction	+	+	+	+	-	-	-	-
Methyl red reaction	-	-	-	-	-	-	-	-
Voges-Proskauer (AMC production)	-	-	-	-	-	-	-	-
Growth on Koser's citrate medium	+	-	+	-	+	+	+	+
Sodium hippurate hydrolysis	+	+	+	+	+	+	+	+
Growth in iron milk	pep	pep	pep	pep	pep	pep	pep	pep
Litmus milk reaction	red	red	red	red	pep	pep	pep	pep
Growth in methylene blue milk	red	red	red	red	red	red	red	red
Urease	+	+	+	+	+	-	+	-
Reduction of anaerobic alk. KNO_3	+	+	+	+	-	-	-	-
Sodium malonate hydrolysis	-	-	-	-	-	-	-	-
Indol production	-	-	-	-	-	-	-	-
Naphthalene salts hydrolysis	+	+	+	+	+	+	+	+
Starch agar hydrolysis	+	+	+	+	+	+	+	+

*Key to notation: - denotes negative
+ denotes positive
red reduced
pep peptonization.
Un-inoculated controls were negative.

Table 10 (Continued)

Test	Cultures							
	#33		#57		7051		8245	
	48	S48	48	S48	48	S48	48	S48
Gelatin agar hydrolysis	+	+	+	+	+	+	+	+
Milk agar hydrolysis	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+
Gram stain	+	+	+	+	+	+	+	+
Growth in nutrient broth								
at 10C	+	+	+	+	-	-	-	-
28C	+	+	+	+	+	+	+	+
30C	+	+	+	+	+	+	+	+
37C	+	+	+	+	+	+	+	+
45C	+	+	+	+	+	+	+	+
55C	-	-	-	-	-	-	-	-
65C	-	-	-	-	-	-	-	-
Growth in filtered D58 medium minus cellulose								
at 10C	+	+	+	+	-	-	-	-
28C	+	+	+	+	+	+	+	+
30C	+	+	+	+	+	+	+	+
37C	+	+	+	+	+	+	+	+
45C	+	+	+	+	+	+	+	+
55C	-	-	-	-	-	-	-	-
65C	-	-	-	-	-	-	-	-

Table 11. Initial transfer and sub-culture (S) data on mesophiles as determined by carbohydrate fermentation reactions in two media; non-synthetic, and synthetic base, at 30C. Observations were made at 72 hr for both the initial transfer and sub-culture. Acid production was checked utilizing brom thymol blue pH indicator*

Fermentation test	Cultures							
	#33		#57		7051		8245	
	72	S72	72	S72	72	S72	72	S72
With non-synthetic base								
Glucose	A	-	A	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Cellulose	-	-	-	-	-	-	-	-
Maltose	A	A	A	A	-	-	-	-
Trehalose	A	-	A	-	-	-	-	-
Sucrose	A	-	A	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-
Fructose	A	A	A	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-
Sodium-β-glycerol-phosphate	-	-	-	-	-	-	-	-

*Key to notation: - denotes negative
A denotes acid production.
Un-inoculated controls were negative.

Table 11 (Continued)

Fermentation test	Cultures							
	#33		#57		7051		8245	
	72	S72	72	S72	72	S72	72	S72
With synthetic base								
Glucose	A	-	A	-	A	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Cellulose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	A	-	-	-
Trehalose	A	-	A	-	A	-	-	-
Sucrose	-	-	A	-	A	-	-	-
Mannitol	-	-	-	-	-	-	-	-
Fructose	A	-	A	-	A	-	-	-
Arabinose	-	-	-	-	-	-	-	-
Sodium-β-glycerol-phosphate	-	-	A	-	-	-	-	-

Table 12. Initial transfer and sub-culture (S) data on mesophiles as determined by slant, and stab cultures on differential media at 30C. Observations were made at 48 hr for both the initial transfer and sub-culture*

Cultural test	Cultures							
	#33		#57		7051		8245	
	48	S48	48	S48	48	S48	48	S48
Soybean agar slant	4	4	4	4	4	4	4	4
Zein agar slant	2	3	2	3	2	3	2	3
Glucose agar slant	4	4	4	4	4	4	4	4
Gelatin stab	3	3	3	3	3	3	1	2
Glucose KNO_3 agar slant	4	4	4	4	4	4	4	4
Nutrient agar slant	4	4	4	4	4	4	4	4
Tyrosine agar slant	4	4	4	4	4	4	4	4
Proteose peptone acid slant	1	-	1	-	-	-	-	-
Glucose yeast extract slant	4	4	4	4	4	4	4	4
Thioglycollate tetrazolium stab	4	4	4	4	4	4	4	4

*Key to notation: - denotes negative
1 denotes poor growth
2 denotes fair growth
3 denotes good growth
4 denotes excellent growth.
Un-inoculated controls were negative.

however, strain ATCC 7051 has growth characteristics that are similar to those of culture #57 in synthetic basal medium.

Growth responses of the mesophilic cultures on various types of agar media (Table 12) are practically identical for all the strains tested; however, cultures #33 and #57 were able to grow on proteose peptone acid agar slants, a characteristic which was not evident for cultures ATCC 7051 and ATCC 8245.

Since cultures #33 and #57 were shown to vary markedly in characteristics from reference cultures B. megaterium ATCC 7051 and ATCC 8245, they were next compared with reference cultures B. licheniformis and B. cereus var. mycoides, according to characteristics of mesophilic bacilli compiled by Knight and Proom (1950).

B. licheniformis can be differentiated from other bacilli by its ability to grow anaerobically and produce gas, when cultured in a mixture of 5% glucose and 20% nutrient gelatin medium at pH 8-9 (Gibson and Abdel-Malek, 1945). This test was determined on culture #33 only, since diagnostic differences between cultures #33 and #57 were not found; therefore, #33 and #57 can be considered to be identical cultures. Culture #33 grew anaerobically, but no gas was

formed from glucose utilization. Since culture #33 gave a negative Gibson and Abdel-Malek reaction, it could not be identified as B. licheniformis.

B. cereus var. mycoides strains are capable of anaerobic growth, and produce a characteristic egg-yolk reaction (Macfarlane et al., 1941). Since culture #33 will grow anaerobically, and was also found to produce a positive egg-yolk reaction, in addition to its agreement with other standard diagnostic tests for B. cereus var. mycoides, the identification process was completed.

Eleven of the diagnostic tests most commonly used for the positive identification of B. cereus var. mycoides were applied to culture #33 after incubation at 30C for 24 hr in kinetin-nutrient broth media containing from 0.00 ug/L of kinetin to 5000.0 ug/L of kinetin (Tables 13 and 14). The results indicate that the levels of kinetin employed did not affect an alteration of the characteristic physiological reactions of B. cereus var. mycoides, #33. To avoid confusion and for convenience in this thesis, the designation #33 will continue to be used with reference to B. cereus var. mycoides.

Table 13. Data on culture #33 pertaining to physiological reactions determined on culture inocula from kinetin-nutrient broth media. Cultures and plates were incubated at 30C for 24 hr*

Level of Kinetin (ug/L) in Nutrient Broth (NB) used to prepare Inocula	Cultural Tests						
	NB pH 9.6	NB 5% NaCl	Koser's Citrate	Anaerob. Alk. KNO_3	Hydrolysis Starch	Milk	Gelatin
0.000	+	+	+	+	+	+	+
0.500	+	+	+	+	+	+	+
1.25	+	+	+	+	+	+	+
2.5	+	+	+	+	+	+	+
5.0	+	+	+	+	+	+	+
10.0	+	+	+	+	+	+	+
20.0	+	+	+	+	+	+	+
40.0	+	+	+	+	+	+	+
80.0	+	+	+	+	+	+	+
160.0	+	+	+	+	+	+	+
320.0	+	+	+	+	+	+	+
640.0	+	+	+	+	+	+	+
1280.0	+	+	+	+	+	+	+
2560.0	+	+	+	+	+	+	+
5000.0	+	+	+	+	+	+	+

*Key to notation: + denotes growth or positive reaction.
Un-inoculated controls were negative.

Table 14. Data on culture #33 pertaining to physiological reactions determined on culture inocula from kinetin-nutrient broth media. Cultures were incubated at 30C for 48 hr*

Level of Kinetin (ug/L) in Nutrient Broth (NB) used to prepare Inocula	Cultural Tests Syn Base Medium			
	KNO_3	Glucose	Arabinose	Xylose
0.000	+	A	-	-
0.500	+	A	-	-
1.25	+	A	-	-
2.5	+	A	-	-
5.0	+	A	-	-
10.0	+	A	-	-
20.0	+	A	-	-
40.0	+	A	-	-
80.0	+	A	-	-
160.0	+	A	-	-
320.0	+	A	-	-
640.0	+	A	-	-
1280.0	+	A	-	-
2560.0	+	A	-	-
5000.0	+	A	-	-

*Key to notation: + denotes growth or positive reaction
A denotes the production of acidic response
- denotes a negative response.
Un-inoculated controls were negative.

Thermophilic cultural characteristics, as evidenced in Tables 15 to 17, were utilized in the identification of the two unknown cultures, TNTM and TC & E. B. stearothermophilus ATCC 7953 and ATCC 7954 do not coincide physiologically with

the unknown thermophilic cultures on the following diagnostic tests (Table 15): growth in nutrient broth containing 2% NaCl; methyl red reaction; growth in anaerobic alkaline KNO_3 medium; hydrolysis of starch, gelatin, and milk; and growth at 45C in nutrient broth medium.

Carbohydrate fermentation data (Table 16) show that none of the cultures tested was able to ferment carbohydrates when they were grown in a non-synthetic basal medium; however, cultures TNTM, ATCC 7953 and ATCC 7954 had identical fermentation patterns when they were grown in a synthetic basal medium. TC & E, in synthetic basal medium, was capable of fermenting only glucose and trehalose of the 10 carbohydrates tested. This culture does not match the carbohydrate fermentation characteristics of TNTM, ATCC 7953 and ATCC 7954, with respect to utilization of maltose and sucrose.

Growth responses of the thermophilic cultures on various types of agar (Table 17) are practically identical for all the strains tested. Slight differences can be noted when the organisms were grown on glucose agar slants and glucose-KNO_3 agar slants.

Major differences in cultural characteristics of TNTM and TC & E, as compared to ATCC 7953 and ATCC 7954, are the

Table 15. Initial transfer and sub-culture data (S) on pure culture thermophiles as determined by diagnostic tests after 48 hr incubation at 65C*

Test	Cultures							
	T NTM		T C & E		7953		7954	
	48	S48	48	S48	48	S48	48	S48
Growth in nutrient broth	+	+	+	+	+	+	+	+
at pH 9.6	-	-	-	-	-	-	-	-
with 2% NaCl	-	-	-	-	+	+	+	+
with 5% NaCl	-	-	-	-	-	-	-	-
KNO_3 reduction	+	+	+	+	+	+	+	+
Methyl red reaction	-	-	-	+	-	+	-	+
Voges-Proskauer (AMC production)	-	-	-	-	-	-	-	-
Growth on Koser's citrate	-	-	-	-	-	-	-	-
Sodium hippurate hydrolysis	+	+	+	+	+	+	+	+
Growth in iron milk	pep	pep	pep	pep	pep	pep	pep	pep
Litmus milk reactions	red	red	red	red	red	red	red	red
Growth in methylene blue milk	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Reduction of anaerobic alk. KNO_3	-	-	+	+	+	+	+	+
Sodium malonate hydrolysis	-	-	-	-	-	-	-	-
Indol production	-	-	-	-	-	-	-	-
Naphthalene salts hydrolysis	+	+	+	-	+	+	+	+
Starch agar hydrolysis	-	-	-	-	+	+	+	+

*Key to notation: - denotes negative
+ denotes positive
pep denotes peptonization
red denotes reduced.
Un-inoculated controls were negative.

Table 15 (Continued)

Test	Cultures							
	T NTM		T C & E		7953		7954	
	48	S48	48	S48	48	S48	48	S48
Gelatin agar hydrolysis	-	-	-	-	+	+	+	+
Milk agar hydrolysis	-	-	-	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+
Growth in nutrient broth								
at 10C	-	-	-	-	-	-	-	-
28C	-	-	-	-	-	-	-	-
30C	-	-	-	-	-	-	-	-
37C	-	-	-	-	-	-	-	-
45C	-	-	-	-	+	+	+	+
55C	+	+	+	+	+	+	+	+
65C	+	+	+	+	+	+	+	+
Growth in filtered D58 medium minus cellulose								
at 10C	-	-	-	-	-	-	-	-
28C	-	-	-	-	-	-	-	-
30C	-	-	-	-	-	-	-	-
37C	-	-	-	-	-	-	-	-
45C	+	+	+	+	+	+	+	+
55C	+	+	+	+	+	+	+	+
65C	+	+	+	+	+	+	+	+

Table 16. Initial transfer and sub-culture data (S) on pure culture thermophiles as determined by carbohydrate fermentation reactions in two media, non-synthetic and synthetic base at 65C. Observations were made at 72 hr for both the initial transfer and sub-culture. Acid production was checked, utilizing brom thymol blue pH indicator*

Fermentation test	Cultures							
	T NTM		T C & E		7953		7954	
	72	S72	72	S72	72	S72	72	S72
With non-synthetic base								
Glucose	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Cellulose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-
Sodium-β-glycerol-phosphate	-	-	-	-	-	-	-	-

*Key to notation: - denotes negative
A denotes acid production.
Un-inoculated controls were negative.

Table 16 (Continued)

Fermentation test	Cultures							
	T NTM		T C & E		7953		7954	
	72	S72	72	S72	72	S72	72	S72
With synthetic base								
Glucose	A	A	A	A	A	A	A	A
Sorbitol	-	-	-	-	-	-	-	-
Cellulose	-	-	-	-	-	-	-	-
Maltose	A	-	-	-	A	-	A	-
Trehalose	A	-	A	-	A	-	A	-
Sucrose	A	-	-	-	A	-	A	-
Mannitol	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-
Sodium-β-glycerol-phosphate	-	-	-	-	-	-	-	-

Table 17. Initial transfer and sub-culture data (S) on pure culture thermophiles as determined by slant and stab cultures on differential media at 65C. Observations were made at 48 hr for both the initial transfer and sub-culture*

Cultural test	Cultures							
	T NTM		T C & E		7953		7954	
	48	S48	48	S48	48	S48	48	S48
Soybean agar slant	3	3	3	4	3	4	3	4
Zein agar slant	1	2	1	-	1	-	1	-
Glucose agar slant	2	4	3	4	2	2	2	2
Gelatin stab	-	-	-	-	-	-	-	-
Glucose KNO_3 agar slant	3	4	3	4	2	3	2	3
Nutrient agar slant	2	2	2	2	2	2	2	2
Tyrosine agar slant	3	4	3	4	3	4	3	4
Proteose peptone acid slant	-	-	-	-	-	-	-	-
Glucose yeast extract slant	4	4	4	4	4	4	4	4
Thioglycollate tetrazolium stab	4	4	4	4	4	4	4	4

*Key to notation: - denotes negative
1 denotes poor growth
2 denotes fair growth
3 denotes good growth
4 denotes excellent growth.
Un-inoculated controls were negative.

inability of the unknown cultures to grow in nutrient broth containing 2% NaCl and their inability to hydrolyze starch, gelatin, and milk agar.

Since B. stearothermophilus is the only described aerobic bacillus capable of growth at 65C (Breed et al., 1957) it was concluded that cultures TNTM and TC & E are variants of B. stearothermophilus. Proposing a new species with regard to the unknown cultures may be premature at this time, as four major cultural characteristics may not be sufficient evidence.

Physiological characteristics of the two mixed cultures, NTM and C & E, are shown in Tables 18 to 20. These two cultures differ slightly in the following characteristics (Table 18): growth in anaerobic alkaline KNO_3 medium, and growth at 45C in nutrient broth medium. Table 19 shows the inability of both of these cultures to ferment carbohydrates when they are grown in a non-synthetic basal medium; however, in a synthetic basal medium, culture NTM ferments maltose and sucrose, whereas culture C & E can only ferment sorbitol. The only noticeable difference between these cultures when they are grown on various types of agar media (Table 20) is the ability of culture C & E to grow on zein agar, whereas

Table 18. Initial transfer and sub-culture data (S) on mixed cultures as determined by diagnostic tests after 48 hr incubation at 65C*

Test	Cultures			
	NTM		C & E	
	48	S48	48	S48
Growth in nutrient broth	+	+	+	+
at pH 9.6	-	-	-	-
with 2% NaCl	-	-	-	-
with 5% NaCl	-	-	-	-
KNO_3 reduction	+	+	+	+
Methyl red reaction	-	-	-	-
Voges-Proskauer (AMC production)	-	-	-	-
Growth on Koser's citrate	-	-	-	-
Sodium hippurate hydrolysis	+	+	+	+
Growth in iron milk	pep	pep	pep	pep
Litmus milk reactions	red	red	red	red
Growth in methylene blue milk	-	-	-	-
Urease	-	-	-	-
Reduction of anaerobic alk. KNO_3	+	+	-	-
Sodium malonate hydrolysis	-	-	-	-
Indol production	-	-	-	-
Naphthalene salts hydrolysis	+	-	+	-
Starch agar hydrolysis	-	-	-	-
Gelatin agar hydrolysis	+	+	+	+
Milk agar hydrolysis	-	-	-	-
Catalase	+	+	+	+
Motility	+	+	+	+

*Key to notation: - denotes negative
+ denotes positive
pep denotes peptonization
red denotes reduced.
Un-inoculated controls were negative.

Table 18 (Continued)

Test	Cultures			
	NTM		C & E	
	48	S48	48	S48
Growth in nutrient broth				
at 10C	-	-	-	-
28C	-	-	-	-
30C	-	-	-	-
37C	-	-	-	-
45C	+	+	-	-
55C	+	+	+	+
65C	+	+	+	+
Growth in filtered D58 medium minus cellulose				
at 10C	-	-	-	-
28C	-	-	-	-
30C	-	-	-	-
37C	-	-	-	-
45C	+	+	+	-
55C	+	+	+	+
65C	+	+	+	+

Table 19. Initial transfer and sub-culture data (S) on mixed cultures as determined by carbohydrate fermentation reactions in two media, non-synthetic and synthetic base at 65C. Observations were made at 72 hr for both the initial transfer and sub-culture. Acid production was checked utilizing brom thymol blue pH indicator*

Fermentation test	Cultures			
	NTM		C & E	
	72	S72	72	S72
With non-synthetic base				
Glucose	-	-	-	-
Sorbitol	-	-	-	-
Cellulose	-	-	-	-
Maltose	-	-	-	-
Trehalose	-	-	-	-
Sucrose	-	-	-	-
Mannitol	-	-	-	-
Fructose	-	-	-	-
Arabinose	-	-	-	-
Sodium-β-glycerol-phosphate	-	-	-	-
With synthetic base				
Glucose	-	-	-	-
Sorbitol	-	-	A	-
Cellulose	-	-	-	-
Maltose	A	-	-	-
Trehalose	-	-	-	-
Sucrose	A	-	-	-
Mannitol	-	-	-	-
Fructose	-	-	-	-
Arabinose	-	-	-	-
Sodium-β-glycerol-phosphate	-	-	-	-

*Key to notation: - denotes negative
A denotes acid production.
Un-inoculated controls were negative.

Table 20. Initial transfer and sub-culture data (S) on mixed cultures as determined by slant and stab cultures on differential media at 65C. Observations were made at 48 hr for both the initial transfer and sub-culture*

Cultural test	Cultures			
	NTM		C & E	
	48	S48	48	S48
Soybean agar slant	4	4	4	4
Zein agar slant	-	-	1	1
Glucose agar slant	3	4	3	4
Gelatin stab	-	-	-	-
Glucose KNO_3 agar slant	2	4	3	4
Nutrient agar slant	2	2	2	2
Tyrosine agar slant	3	4	4	4
Proteose peptone acid slant	-	-	-	-
Glucose yeast extract slant	4	4	4	4
Thioglycollate tetrazolium stab	4	4	4	4

*Key to notation: - denotes negative
1 denotes poor growth
2 denotes fair growth
3 denotes good growth
4 denotes excellent growth.
Un-inoculated controls were negative.

culture NTM lacks this ability.

Since cultures NTM and C & E refer to cellulose digesting mixed cultures which were obtained by combining, in the case of NTM; #33 and TNTM, and, in the case of C & E; #57 with TC & E, it is interesting to compare the cultural character-

istics of each of the pure culture mesophiles and thermophiles with their respective mixed cultures, namely NTM and C & E (Tables 10 to 12, and Tables 15 to 20).

In nutrient broth, the mesophiles grow well between 28-37C and poorly at 10C and 45C. The mesophile-thermophile mixed cultures and the pure culture thermophiles grow only between 55C and 65C, with the exception of the NTM mixture which grows poorly at 45C.

Hydrolytic characteristics are noted for the mesophiles on starch, gelatin, and milk agar, whereas the mixed mesophile-thermophile cultures are negative on starch and milk agar media, but they possess the ability to hydrolyze gelatin on initial transfer and sub-culture, which the pure culture thermophiles are unable to do.

Citrate can be utilized by the mesophiles on initial transfer, but they cannot withstand sub-culturing in this medium. As noted in Tables 15 and 18, neither the pure culture thermophiles nor the mixed mesophile-thermophile cultures show a positive growth response in Koser's citrate medium.

Naphthalene salts medium supports the growth of all the test organisms on initial transfer except for TC & E, but the mixed mesophile-thermophile cultures fail to respond on sub-

culture in this medium.

Glucose and trehalose are readily fermented by the mesophiles and thermophiles on initial transfer in synthetic basal medium; however, only the thermophiles will sub-culture in glucose synthetic medium. Cultures NTM and TNTM produce acid in maltose and sucrose on initial transfer, but they fail to sub-culture on these media. In the cases of glucose and trehalose fermentation, the pure cultures of the mesophiles and thermophiles demonstrate an acidic response while the two symbiotic mixed cultures are negative.

Apparently there are several interactions between these mesophiles and thermophiles, as noted above, which cause various alterations in the biochemical patterns of the mixed cultures, NTM and C & E.

Statistical Analyses of Fermentation Data

The effect of sterile culture filtrates on fermentative activities of cultures #33 and #57

In order to compare the effects of filter sterilized C & E culture filtrate and sterile D58 medium filtrate on the two mesophilic strains, #33 and #57, a 2 by 2 by 5 (2 X 2 X 5) factorial experiment was designed for statistical analysis.

In this experiment, the fermentation responses of 2 organisms grown in 2 types of carbohydrate media were determined. Each medium was tested with 5 levels of culture filtrate (Table 21).

The analysis of variance (ANOV) for these data is shown in Table 22. Carbohydrates (C), filtrates (F), and the C X F interaction were found to be significant at the .01 level of probability; therefore, significant differences are indicated in this test, between the two carbohydrates tested, cellobiose and glucose, and similar differences are shown to exist between the filtrates tested. In order to determine the amount of variation contributed by each specific filtrate tested, to the total amount of filtrate variation, the sum of squares for filtrates was partitioned into four meaningful orthogonal comparisons. The results indicated that a significant difference at the .01 level of probability does exist when (a) the control is contrasted with levels of filtrate, (b) C & E filtrate is compared with D58 filtrate, (c) the 10% concentrations of both filtrates are compared with the 50% concentrations of filtrates, and (d) the upper and lower extremes of both filtrate concentrations are compared against each other. These trends suggest a graded response to factors in yeast extract, and the production of

Table 21. Data from two replications of the 2 X 2 X 5 factorial experiment. Data are recorded as number of ml of 0.001N NaOH required to titrate samples to pH 7.0. Cultures were incubated at 30C for 5 days

Carbohydrate (C)	Filtrate (F)	Organism (O)	Rep. 1		Rep. 2		Trt Total
			1	2	1	2	
Cellobiose	None (A)	33	6.5	6.8	0.0	0.0	13.3
		57	6.5	7.9	0.0	0.0	14.4
	10% C & E (B)	33	14.3	16.6	16.1	16.7	63.7
		57	23.0	21.0	16.9	16.6	77.5
	50% C & E (C)	33	76.2	70.1	85.7	90.2	322.2
		57	80.2	81.6	92.3	95.8	349.9
	10% D58 (D)	33	9.7	6.0	12.7	10.5	38.9
		57	5.9	6.2	11.8	11.9	35.8
	50% D58 (E)	33	13.2	11.0	28.7	30.7	83.6
		57	14.0	8.9	34.2	31.8	88.9
Glucose	None (A)	33	7.0	12.7	0.0	0.0	19.7
		57	14.3	12.3	14.0	11.8	52.4
	10% C & E (B)	33	17.5	14.0	16.6	14.6	62.7
		57	21.0	23.0	16.0	16.8	76.8
	50% C & E (C)	33	83.6	83.7	94.0	96.5	357.8
		57	95.0	94.1	91.5	93.7	374.3

Table 21 (Continued)

Carbohydrate (C)	Filtrate (F)	Organism (O)	Rep. 1		Rep. 2		Trt Total
			1	2	1	2	
	10% D58 (D)	33	16.4	17.0	22.6	19.6	75.6
		57	17.0	18.2	19.9	19.1	74.2
	50% D58 (E)	33	55.9	52.1	65.0	63.9	236.9
		57	63.8	57.4	62.0	66.4	249.6
Total			641.0	620.6	700.0	706.6	
			1261.6		1406.6		2668.2

Table 22. Analysis of variance of 2 X 2 X 5 data. Values obtained by performing F tests are shown

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications (R)	1	274.31	274.31	
Carbohydrates (C)	1	3,023.34	3,023.34	38.62**
Filtrates (F)	4	70,084.57	17,521.14	223.80**
A vs BCDE	(1)	(14,704.46)	14,704.46	187.82**
BC vs DE	(1)	(10,035.03)	10,035.03	128.18**
BD vs CE	(1)	(37,927.56)	37,927.56	484.45**
BE vs CD	(1)	(7,417.52)	7,417.52	94.74**
Cultures (O)	1	178.21	178.21	2.28
C X F	4	3,839.81	959.95	12.26**
C X O	1	11.10	11.10	0.14
F X O	4	85.47	21.37	0.27
C X F X O	4	62.75	15.69	0.20
Exptl Error	19	1,487.60	78.29	
Sampling Error	40	152.12	3.80	
Total	79	79,187.78		

**Indicates significance at the .01 level of probability.

materials with like effect by growing C & E cultures at 65C.

Cultures (O), C X O, F X O, and C X F X O interactions were found to contribute a non-significant amount of variation in this experiment.

The C X F interaction was found to be significant at the .01 level of probability, which seemed to suggest that the stimulatory filtrates were not behaving in the same manner with each carbohydrate tested. Therefore, these results were analyzed from a more detailed statistical viewpoint.

Data for each carbohydrate (Table 21) were analyzed separately (Tables 23 and 24) to confirm or refute the significant effect of added filtrates as noted in Table 22. The separate carbohydrate analyses indicated that the effect of filtrates was significant at the .01 level of probability, including the four orthogonal comparisons. Cultures (O) and the F X O interaction were found to contribute a non-significant amount of variation in both analyses. The experimental error, which represents the inherent variability of the experimental material, was higher for the cellobiose data than it was for the glucose data; however, in both instances the error due to sampling was at a minimum.

Since the C X F interaction (Table 22) was significant

at the .01 level of probability when tested with the experimental error mean square of 78.29, it was felt that the sum of squares for the latter term should be partitioned into its component parts to determine the actual amount of variability contributed by C X F X R. All of the mean squares shown in Table 25 are better estimates of the actual variance contributed by each of the respective components to the total experimental error. For these comparisons, the F values shown in Table 25 were obtained by dividing each of the partitioned mean squares into their respective mean squares shown in Table 22. The new F value for C X F (Table 25) is approximately twice as high as the F value calculated in Table 22. This result clearly substantiates the significance of the C X F interaction.

It can be concluded from the analyses of the data from this experiment that (1) culture filtrates, most noticeably 50% concentration of C & E filtrate, are capable of stimulating growth responses of cultures #33 and #57; and (2) there is a significant amount of variation in the experiment attributable to the specific carbohydrate tested and the C X F interaction. The latter point can best be explained by a noticeable preference of the organisms for glucose as a

Table 23. Analysis of variance pertaining to cellobiose data in the 2 X 2 X 5 factorial experiment

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications (R)	1	342.23	342.23	
Filtrates (F)	4	33,865.46	8,466.37	76.49**
A vs BCDE	(1)	(5,637.06)	5,637.06	50.93**
BC vs DE	(1)	(10,014.66)	10,014.66	90.48**
BD vs CE	(1)	(12,351.99)	12,351.99	111.60**
BE vs CD	(1)	(5,861.74)	5,861.74	52.96**
Cultures (O)	1	50.18	50.18	0.45
F X O	4	74.40	28.60	0.26
Exptl Error	9	996.12	110.68	
Sampling Error	20	71.35	3.57	
Total	39	35,399.74		

**Indicates significance at the .01 level of probability.

Table 24. Analysis of variance pertaining to glucose data in the 2 X 2 X 5 factorial experiment

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications (R)	1	19.60	19.60	
Filtrates (F)	4	40,058.92	10,014.73	229.64**
A vs BCDE	(1)	(9,294.88)	9,294.88	213.14**
BC vs DE	(1)	(1,730.19)	1,730.19	39.67**
BD vs CE	(1)	(26,987.45)	26,987.45	618.84**
BE vs CD	(1)	(2,046.40)	2,046.40	46.93**
Cultures (O)	1	139.13	139.13	3.19
F X O	4	73.82	18.46	0.14
Exptl Error	9	392.46	43.61	
Sampling Error	20	80.77	4.04	
Total	39	40,764.70		

**Indicates significance at the .01 level of probability.

Table 25. Analysis of variance of partitioned experimental error sum of squares obtained from data in 2 X 2 X 5 factorial experiment

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values[a]
Exptl Error	19	1,487.60	78.29	
C X R	(1)	(99.02)	99.02	13.05
F X R	(4)	(1,031.45)	257.86	16.79**
O X R	(1)	(22.68)	22.68	7.86
C X F X R	(4)	(148.61)	37.15	25.84**
C X O X R	(1)	(13.61)	13.61	0.82
F X O X R	(4)	(90.96)	22.74	0.94
C X F X O X R	(4)	(81.27)	20.32	0.77
Total	19	1,487.60		

[a]F values were obtained by dividing each of the partitioned mean squares into their respective mean square calculated in the pooled ANOV (Table 22).

**Indicates significance at the .01 level of probability.

fermentable carbohydrate over cellobiose. Both of these carbohydrates are utilizable; however, cultures #33 and #57 may more readily ferment or degrade a monosaccharide than a disaccharide.

Fermentative activity of culture #33 with selected carbohydrates

A randomized block design was utilized to detect differences between carbohydrates in supporting the stimulatory fermentation response of culture #33 when 50% C & E culture filtrate was added to carbohydrate media. Three carbohydrate energy sources were selected to compare their relative rates of utilization by culture #33. One hexose, supplied by glucose medium; one pentose, supplied by xylose medium; and one oligosaccharide, supplied by trehalose medium, were compared with a control medium containing no carbohydrate at all. The data (Table 26) for two replications of the experiment point out that glucose (G) was utilized to the highest extent. The control (N) and xylose (X) were both minimal in supporting acid formation, thereby emphasizing the fact that a carbohydrate known to be non-utilizable by culture #33 (Table 14) is not transformed to a utilizable form by the addition of 50% C & E culture filtrate.

Table 26. Data from two replications of the randomized block experiment designed to determine differences between carbohydrates in supporting the stimulatory effect produced by adding 50% C & E culture filtrate to fermentation media inoculated with culture #33. Data are recorded as number of ml of 0.001N NaOH required to titrate samples to pH 7.0. Cultures were incubated at 30C for 48 hr

Replication	Carbohydrate				
	None	Glucose	Xylose	Trehalose	Total
1	35.4	88.4	36.0	55.2	
	35.8	86.6	33.2	63.2	
	35.1	88.8	35.8	61.5	
	35.7	89.0	35.4	70.0	
	142.0	352.8	140.4	249.9	885.1
2	34.3	90.8	37.7	76.5	
	36.2	89.8	36.6	71.6	
	37.0	92.8	35.4	73.3	
	35.8	91.7	36.0	74.0	
	143.3	365.1	145.7	295.4	949.5
Total	285.3	717.9	286.1	545.3	1,834.6

Trehalose (T) and glucose (G), known to be utilizable by culture #33 (Table 11), were found to be significantly different, at the .01 level of probability, in demonstrating the stimulatory effect on culture #33 as evidenced by the ANOV (Table 27).

Since glucose was most readily fermentable by culture

#33 as compared to either cellobiose (Table 21) or trehalose, glucose was chosen as the standard carbohydrate source for the remainder of the study.

The effect of source of culture filtrate on the fermentative activity of culture #33

A randomized block design was employed to detect differences between various sources of culture filtrate for demonstrating a stimulatory effect on culture #33. Data relative to the effect of adding culture filtrate from D58 medium, cultures #33, TC & E, and C & E are shown in Table 28.

Results indicate that filtrates from D58 medium and culture TC & E are equally capable of supplying the necessary growth factor required to demonstrate a stimulatory response by culture #33. However, C & E culture filtrate seems to supply the greatest amount of growth factor as evidenced by a higher response with this culture filtrate than with any of the others tested.

The ANOV of these data (Table 29) shows that the filtrate treatment effect is significant at the .01 level of probability. The orthogonal comparisons which were carried out show that significant differences do exist between the

Table 27. Analysis of variance of carbohydrate fermentation data. Values obtained by performing F tests are shown

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications	1	129.61	129.61	
Treatments	3	16,817.81	5,605.94	110.79**
N vs GXT	(1)	(5,008.37)	5,008.37	98.98**
GT vs X	(1)	(9,947.52)	9,947.52	196.59**
G vs T	(1)	(1,861.92)	1,861.92	36.80**
Exptl Error	3	151.81	50.60	
Sampling Error	24	143.98	6.00	
Total	31	17,243.21		

**Indicates significance at the .01 level of probability.

Table 28. Data from the randomized block experiment designed to determine differences in filtrate sources. Data are recorded as number of ml of 0.001N NaOH required to titrate samples to pH 7.0. Tubes were incubated at 30C for 48 hr

Replication	Filtrate (50% v/v) D58 (A)	#33 (B)	TC & E (C)	C & E (D)	Total
1	56.6	65.3	54.3	79.0	
	54.3	66.2	50.0	80.7	
	55.3	67.3	52.0	84.3	
	57.6	65.7	56.6	81.2	
	223.8	264.5	212.9	325.2	1,026.4
2	57.6	68.0	47.9	93.6	
	55.2	65.2	48.3	92.0	
	58.1	68.2	49.4	96.8	
	59.3	67.9	46.6	90.5	
	230.2	269.3	192.2	372.9	1,064.6
3	56.8	64.5	58.0	77.7	
	58.5	63.2	58.3	78.0	
	59.3	65.2	59.0	75.0	
	58.2	64.0	57.6	77.4	
	232.8	256.9	232.9	308.1	1,030.7
Total	686.8	790.7	638.0	1,006.2	3,121.7

Table 29. Analysis of variance of culture filtrate data. Values obtained by performing F tests are shown

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications	2	54.73	27.37	
Treatments	3	6,677.54	2,225.85	17.89**
A vs BCD	(1)	(973.96)	973.96	7.83*
BD vs C	(1)	(3,768.57)	3,786.57	30.29**
B vs D	(1)	(1,935.01)	1,935.01	15.55**
Exptl Error	6	746.52	124.42	
Sampling Error	36	100.54	2.79	
Total	47	7,579.33		

*Indicates significance at the .05 level of probability.

**Indicates significance at the .01 level of probability.

sources of filtrate. Of these, the C & E culture filtrate was found to contribute the greatest amount of variation when tested at the .01 level of probability.

Therefore, it can be concluded from the analysis of these data that a significant amount of growth factor is present in D58 medium only after growth of the C & E mixed

culture has taken place. As a result of this finding, C & E mixed culture filtrates were used as a standard in comparing similar results obtained with other test systems.

The effect of kinetin on the fermentative activity of culture #33

Data obtained from two replications of the experiment designed to determine the effect of kinetin on growth of culture #33 in 0.5% glucose-phosphate buffer media are recorded in Table 30. The mean values for replication 1, replication 2, and the pooled data are shown in Figure 1. The graph of the data illustrates the relatively harmonic curves obtained with increasing concentrations of kinetin. Phosphate buffer at two concentrations, 1/2X and 1X, merely shifts the total curve to the right in the case of 1X buffer concentration as compared to 1/2X buffer concentration.

The ANOV of these data is presented in Table 31. Since preliminary data on the effect of kinetin on culture #33 indicated that a reliable assay for kinetin could not be developed, these data were analyzed as trend comparisons. Orthogonal coefficients utilized in determining the sums of squares for the kinetin trend comparisons were compiled by Anderson and Houseman (1942).

Table 30. Data obtained from two replications of the experiment designed to determine the effect of kinetin on growth of culture #33 in 0.5% glucose fermentation media employing two levels of buffer concentration. Data are recorded as number of ml of 0.001N NaOH required to titrate samples to pH 7.0. Cultures were incubated at 30C for 7 days

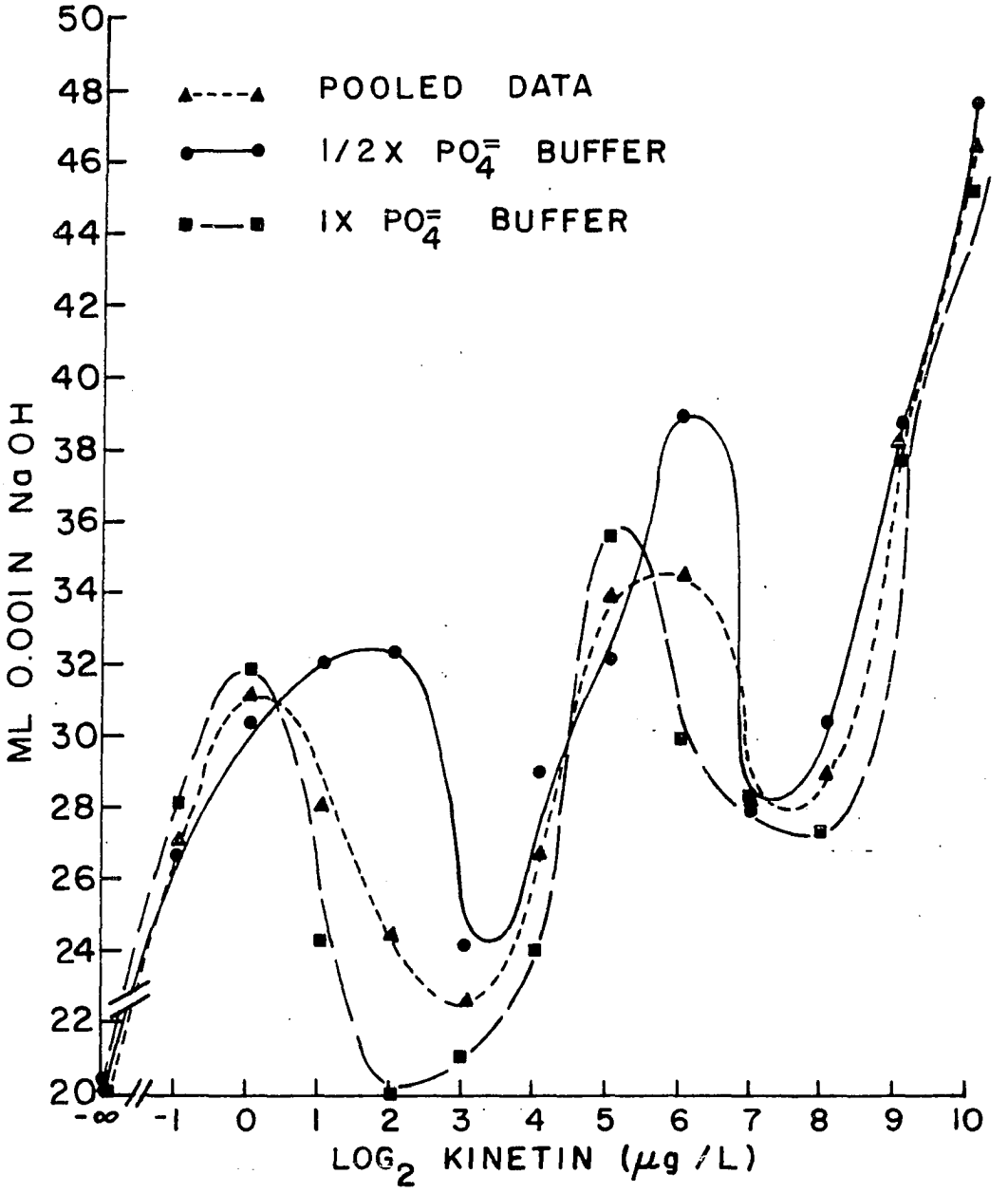
Kinetin (K)* (ug/L)	Buffer Concentration (B) 1/2X Replication 1	2	Total	1X Replication 1	2	Total
0.000	18.8	23.6		29.9	10.8	
	17.2	21.9		30.3	8.6	
	36.0	45.5	81.5	60.2	19.4	79.6
0.50	23.0	27.5		38.0	20.0	
	26.6	29.3		35.9	18.2	
	49.6	56.8	106.4	73.9	38.2	112.1
1.00	27.6	33.8		34.4	32.2	
	28.0	31.3		31.5	29.5	
	55.6	65.1	120.7	65.9	61.7	127.6
2.00	29.8	33.5		20.9	28.9	
	28.6	35.6		21.1	26.2	
	58.4	69.1	127.5	42.0	55.1	97.1
4.00	24.2	36.5		15.0	15.7	
	29.8	38.8		16.3	18.9	
	54.0	75.3	129.3	31.3	34.6	65.9
8.00	14.9	34.5		19.1	20.8	
	14.6	32.6		21.6	22.6	
	29.5	67.1	96.6	40.7	43.4	84.1

*Adjusted to integers of $\log_2$ values excluding 1000 ug/L level.

Table 30 (Continued)

Kinetin (K) (ug/L)	Buffer Concentration (B) 1/2X Replication 1	2	Total	1X Replication 1	2	Total
16.00	24.0	29.9		22.2	26.8	
	34.4	28.7		19.6	27.5	
	58.4	58.6	117.0	41.8	54.3	96.1
32.00	36.4	27.9		32.2	39.2	
	38.9	25.4		28.3	42.1	
	75.3	53.3	128.6	60.5	81.3	141.8
64.00	37.1	40.2		15.0	46.3	
	39.8	38.7		14.8	43.5	
	76.9	78.9	155.8	29.8	89.8	119.6
128.00	25.6	29.1		14.0	43.3	
	26.3	30.8		15.5	41.0	
	51.9	59.9	111.8	29.5	84.3	113.8
256.00	24.8	36.1		17.0	38.8	
	25.2	35.0		16.8	36.7	
	50.0	71.1	121.1	33.8	75.5	109.3
512.00	42.7	36.3		33.5	43.0	
	40.2	35.4		32.3	41.0	
	82.9	71.7	154.6	65.8	84.0	149.8
1000.00	56.5	38.5		47.4	44.0	
	56.7	37.9		46.3	42.6	
	113.2	76.4	189.6	93.7	86.6	180.3
Total:	Rep 1 1460.6					
	Rep 2 1657.0					3117.6

Figure 1. Plot of the mean values for replication 1, replication 2, and the pooled data for the experiment designed to determine the effect of kinetin on culture #33 when grown in glucose fermentation media containing two levels of phosphate buffer concentration. Kinetin concentrations are shown as integer $\log_2$ values, e.g. 2^n = ug/L, where n assumes the values shown on the abscissa


POOLED DATA
1/2X $PO_4^=$ BUFFER
1X $PO_4^=$ BUFFER
ML 0.001N NaOH
50
48
46
44
42
40
38
36
34
32
30
28
26
24
22
20
-∞
-1
0
1
2
3
4
5
6
7
8
9
10
LOG_2 KINETIN (μg/L)

As shown in the ANOV (Table 31), buffer (B) was found to be non-significant as was the buffer by kinetin (B X K) interaction. Therefore, buffer is not of significant consequence in demonstrating the effect of kinetin, according to these data. Kinetin concentration, on the other hand, was found to contribute a significant amount of variation when tested at the ,05 level of probability. By partitioning the sum of squares due to the kinetin effect, orthogonal trend comparisons were obtained in which the kinetin effect was found to be significant at the .01 level of probability with respect to a linear kinetin trend, and significant at the .05 level of probability with respect to a quintic kinetin trend. When this experiment was conducted with an expanded range of kinetin levels, 0.00 ug/ml to 50 ug/ml, a similar quintic response was noted for culture #33 as that shown in the ANOV (Table 31).

In order to build a model for prediction purposes, one must determine the order of polynomial which best fits the data. When the data do not fit the standard linear regression model, higher degree polynomials must be incorporated into a valid statistical analysis. By utilizing a trend comparison, one can determine the most appropriate fit of

Table 31. Analysis of variance of data pertaining to the kinetin effect on fermentation patterns of culture #33

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications	1	370.90	370.90	
Buffer (B)	1	256.73	256.73	1.64
Kinetin (K)	12	4,592.31	382.69	2.44*
K_{Linear}	(1)	(2,385.82)	2,385.82	15.23**
$K_{Quadratic}$	(1)	(417.74)	417.74	2.67
K_{Cubic}	(1)	(508.89)	508.89	3.25
$K_{Quartic}$	(1)	(0.13)	0.13	0.0008
$K_{Quintic}$	(1)	(722.45)	722.45	4.61*
Deviations	(7)	(557.28)	79.61	0.51
B X K	12	663.01	55.25	0.35
Exptl Error	25	3,916.66	156.67	
Sampling Error	52	167.28	3.22	

*Indicates significance at the 0.05 level of probability.

**Indicates significance at the 0.01 level of probability.

the data analyzed. However, sampling variation is subject to change and the model for the entire population might best be described by a very complex mathematical expression instead of a quintic degree polynomial which serves as an approximation of the true model.

Since the quintic degree of polynomial was found to contribute a significant amount of variation in describing the kinetin effect, one can postulate on possible mechanisms within the biological material which tend to explain the observed response.

Mechanistic theories pertaining to the action of kinetin on cells are relatively non-existent. However, one could postulate that in this test system more than one enzyme involved in the metabolism of glucose by culture #33 is responsive to the effect of kinetin. The different enzyme systems would tend to be affected to varying degrees as evidenced by the effect of level of kinetin (Figure 1). Kulayeva and Vorob'yeva (1962) assume that kinetin increases the rate of formation of protein, inducing accumulation in the cells of material for its structure. This can take place because kinetin enhances the flow of available forms of nitrogen from the surrounding medium. In addition to such

an indirect effect, they propose that a direct effect of kinetin may be on the formation or primary activation of amino acids.

Until more conclusive evidence is provided, workers can merely postulate upon possible mechanisms of kinetin action.

The effect of kinetin on the fermentative activity of culture TC & E

Data obtained from two replications of the experiment designed to determine the effect of kinetin on growth of culture TC & E in 0.5% glucose-phosphate buffer media are recorded in Table 32. The mean values for replication 1, replication 2, and the pooled data are shown in Figure 2. The graph of the data illustrates the inhibitory trend of kinetin on culture TC & E. Since three levels of kinetin, 2.5, 10, and 160 ug/L that were used in the carbohydrate medium failed to yield results on culture TC & E in replication 2 as compared to replication 1, missing values were calculated (Snedecor, 1956) for use in the analysis.

The ANOV of this set of data (Table 33) showed that kinetin concentration contributed a non-significant amount of variation to the experiment. However, trend comparisons illustrated that the data fitted a linear response curve

Table 32. Pooled data from two replications of the experiment designed to determine the effect of kinetin concentration on growth of culture TC & E in 0.5% glucose fermentation media. Data shown are unweighted means of five observations per kinetin level per replication. Data are recorded as number of ml of 0.001N NaOH required to titrate samples to pH 7.0. Cultures were incubated at 65C for 7 days

Kinetin (K)* (ug/L)	Replication 1	Replication 2	Total
0.00	47.5	65.5	113.0
0.60	49.4	56.9	106.3
1.25	42.0	59.1	101.1
2.50	44.0	63.6**	107.6
5.00	45.3	55.9	101.2
10.00	51.5	71.1**	107.9
20.00	34.1	60.0	94.1
40.00	37.1	62.2	99.3
80.00	30.5	58.8	89.3
160.00	35.4	55.0**	90.4
320.00	35.8	57.9	93.7
640.00	37.8	63.4	101.2

*Denotes calculated missing value.

**Adjusted to integers of $\log_2 K - \log_2 5$ excluding the two highest kinetin levels.

Table 32 (Continued)

Kinetin (K) (ug/L)	Replication 1	Replication 2	Total
1,280.00	23.1	59.9	83.0
2,560.00	37.0	53.9	90.9
5,000.00	36.5	51.2	87.7
50,000.00	27.7	35.3	63.0
Total	614.7	929.7	1,544.4

Figure 2. Plot of the mean values for replication 1, replication 2, and the pooled data for the experiment designed to determine the effect of kinetin on culture TC & E when grown in glucose fermentation media. Kinetin concentrations are shown as integer $\log_2 K - \log_2 5$ values, e.g. $5(2^n)$ = ug/L, where n assumes the values shown on the abscissa

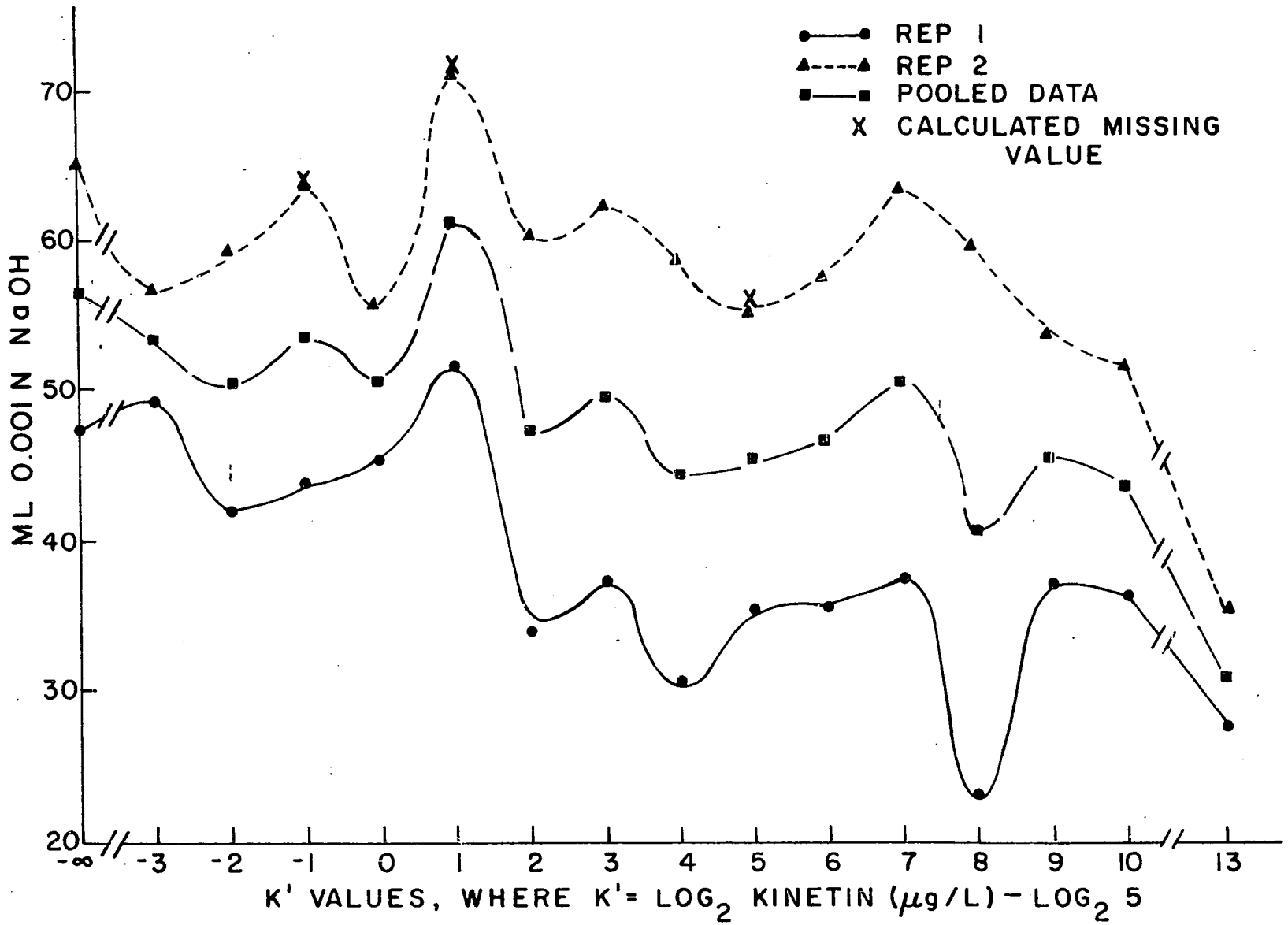

REP 1
REP 2
POOLED DATA
X CALCULATED MISSING VALUE
ML 0.001N NaOH
70
60
50
40
30
20
-∞
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
13
K' VALUES, WHERE K'= LOG2 KINETIN (μg/L) − LOG2 5

Table 33. Analysis of variance of data pertaining to the kinetin effect on fermentation patterns of culture TC & E

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications	1	3,100.79	3,100.79	
Kinetin (K)	15	1,380.92	92.06	2.47
K_{Linear}	(1)	(827.20)	827.20	22.18**
$K_{Quadratic}$	(1)	(50.67)	50.67	1.36
K_{Cubic}	(1)	(27.65)	27.65	0.74
$K_{Quartic}$	(1)	(43.14)	43.14	1.16
$K_{Quintic}$	(1)	(117.47)	117.47	3.15
Deviations	(10)	(314.79)	31.48	0.84
Exptl Error	12[a]	447.55	37.30	
Total	28[a]	4,929.26		

[a]Three degrees of freedom were subtracted from the total as three missing values were utilized in deriving sum of squares for the analysis.

**Indicates significance at the 0.01 level of probability.

since this component was significant at the .01 level of probability. The quadratic, cubic, quartic, and quintic responses were found to be non-significant as were the deviations from regression.

Thus, it can be concluded from the analysis of these data that kinetin concentration does not have a significant effect on the fermentation response of culture TC & E; however, the regression line which was determined is following a linear decreasing trend.

The effect of adding filtrate from kinetin, and yeast extract containing media to fermentation media

Data obtained from two replications of the experiment designed to determine differences in growth stimulation of culture #33 caused by the addition of filtrates from kinetin, and yeast extract media to fermentation media are recorded in Table 34. Sums of three observations per replication were totalled for each of the three v/v filtrate concentrations. Capital letters were assigned to each of these sums for use in the ANOV shown in Table 35. Mean values for each letter designation are shown in the form of a graph (Figure 3).

As noted in the ANOV (Table 35), treatment effect contributed a significant amount of variation when tested at the .01 level of probability. The partitioned sum of squares

Table 34. Data obtained from two replications of the experiment designed to determine differences in growth stimulation of culture #33 caused by the addition of filtrates from kinetin and yeast extract media to fermentation media. Data are recorded as number of ml of 0.001N NaOH required to titrate samples to pH 7.0. Cultures were incubated at 30C for 7 days

Medium	Final Filtrate Concentration								
	0%			10%			50%		
	Rep 1	Rep 2	Total	Rep 1	Rep 2	Total	Rep 1	Rep 2	Total
Control	17.6	19.2							
	19.5	12.9	(A)		-			-	
	16.7	15.0							
	53.8	47.1	100.9						
#1				19.9	11.3		88.0	84.3	
(D58)		-		18.7	9.3	(B)	88.7	88.2	(G)
				19.3	13.4		83.9	89.5	
				57.9	34.0	91.9	260.6	262.0	522.6
#2				17.6	29.3		123.0	124.0	
(D58 plus NaGP)		-		20.4	24.9	(C)	122.7	127.0	(H)
				21.4	23.7		125.5	130.3	
				59.4	77.9	137.3	371.2	381.3	752.5

Table 34 (Continued)

Medium	Final Filtrate Concentration								
	0%			10%			50%		
	Rep 1	Rep 2	Total	Rep 1	Rep 2	Total	Rep 1	Rep 2	Total
#3				17.1	22.8		64.5	72.0	
(D58 minus	-			17.7	20.3	(D)	67.2	60.5	(I)
YE and NaGP)				15.5	21.7		52.2	71.5	
				50.3	64.8	115.1	183.9	204.0	387.9
#4				19.7	24.7		104.9	95.0	
(Kinetin)	-			24.6	23.8	(E)	94.0	98.1	(J)
				17.7	24.5		76.4	86.7	
				62.0	73.0	135.0	275.3	279.8	555.1
#5				20.6	33.9		67.2	70.2	
(Kinetin-free)	-			22.0	30.1	(F)	87.6	76.8	(K)
				19.0	31.4		78.1	74.6	
				61.6	95.4	157.0	232.9	221.6	454.5

Figure 3. Plot of pooled data from two replications of the experiment designed to determine differences in growth stimulation of culture #33 caused by the addition of filtrates from various media to fermentation media

Key: Source of filtrate

#1 D58 medium

#2 D58 plus sodium glycerophosphate medium

#3 D58 medium minus yeast extract and sodium glycerophosphate

#4 Kinetin medium

#5 Kinetin-Free medium

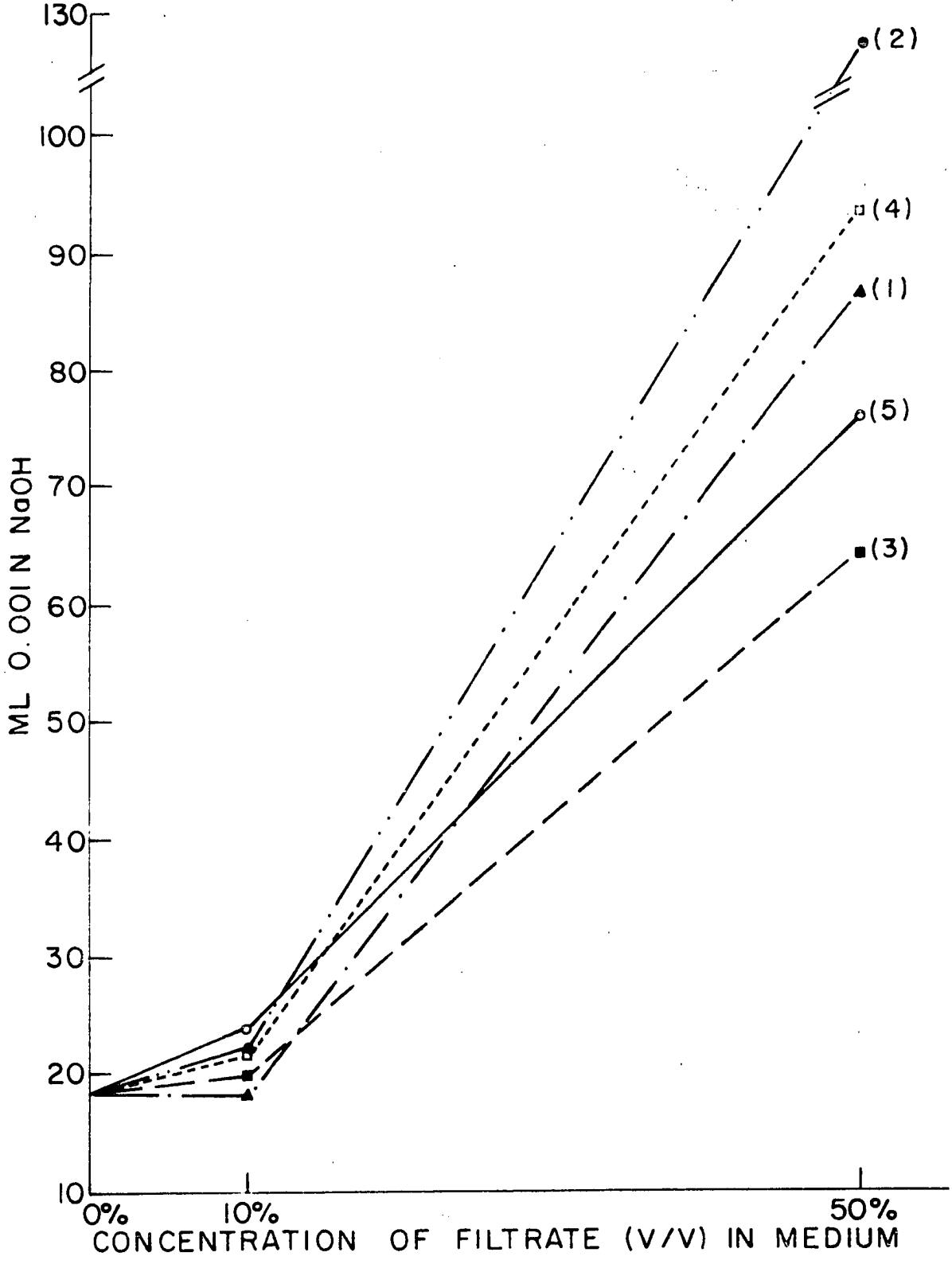

130
100
90
80
70
60
50
40
30
20
10
ML 0.001 N NaOH
(2)
(4)
(1)
(5)
(3)
0%
10%
50%
CONCENTRATION OF FILTRATE (V/V) IN MEDIUM

Table 35. Analysis of variance of data pertaining to the effect of adding filtrates from kinetin and yeast extract media to fermentation media inoculated with culture #33

Source of variation	Degrees of freedom	Sum of squares	Mean square	F values
Replications	1	78.55	78.55	
Treatments	10	90,193.90	9,019.39	206.82**
A vs Others	(1)	(8,014.45)	8,014.45	183.78**
B-F vs G-K	(1)	(69,108.63)	69,108.63	1,584.70**
D vs BCEF	(1)	(30.81)	30.81	0.71
I vs GHJK	(1)	(4,478.63)	4,478.63	102.70**
F vs BCE	(1)	(158.42)	158.42	3.63
K vs GHJ	(1)	(3,025.12)	3,025.12	69.37**
C vs BE	(1)	(63.20)	63.20	1.45
H vs GJ	(1)	(5,071.81)	5,071.81	116.30**
B vs E	(1)	(154.80)	154.80	3.55
G vs J	(1)	(88.02)	88.02	2.02
Exptl Error	10	436.12	43.61	
Sampling Error	44	1,081.41	24.58	
Total	65	91,789.98		

**Indicates significance at the 0.01 level of probability.

for treatments yielded ten orthogonal comparisons. Significant amounts of variation at the .01 level of probability were contributed by the following comparisons: (1) 0% filtrate concentration compared with 10% and 50% concentrations, (2) 10% filtrate concentrations versus 50% concentrations, (3) 50% concentrations of filtrate containing yeast extract, kinetin and/or sodium glycerophosphate versus 50% concentration of filtrate containing none of the above, (4) 50% concentrations of filtrate containing yeast extract, sodium glycerophosphate, and/or kinetin versus 50% concentration of filtrate containing sodium glycerophosphate, and (5) 50% concentration of filtrate containing yeast extract and sodium glycerophosphate versus 50% concentrations of filtrates containing yeast extract or kinetin and sodium glycerophosphate. Non-significant amounts of variation were contributed by comparisons involving 10% concentrations of all the filtrates tested. However, the final comparison made, 50% D58 medium filtrate versus 50% kinetin medium filtrate, was also non-significant when tested at the .01 level of probability. Therefore, it can be concluded from the analysis of these data that 50% concentrations of filtrate are significantly different from 10% concentrations

of filtrate in supporting the stimulatory effect on culture #33; of the 50% concentrations of filtrate tested, filtrate from a medium containing both yeast extract and sodium glycerophosphate was found to be the most stimulatory; the effects of filtrate from D58 medium and the kinetin medium were non-significantly different in demonstrating the enhanced growth response of culture #33. Thus, it appears that kinetin and sodium glycerophosphate are capable of substituting for growth factors found in yeast extract when tested with culture #33.

SUMMARY

Culture C & E, a mixed mesophile-thermophile culture which symbiotically digests cellulose at 65C, has been identified as Bacillus cereus var. mycoides, in the case of the mesophilic symbiont, #33, and a variant of B. stearothermophilus, in the case of the thermophilic symbiont, TC & E.

Quinn (1949) reported that yeast extract was the richest source of factors required for growth and cellulose digestion by the C & E mixed culture at 65C in a cellulose-salts medium, which he designated as D58 medium. It has been found that kinetin (1 ug/L) and sodium glycerophosphate (6 g/L) are capable of substituting for factors in yeast extract in supporting cellulose digestion by the C & E mixed culture at 65C.

It was demonstrated that associative growth of the mesophile and thermophile yielded a factor that is stimulatory to glucose fermentation by the mesophile. This effect was noted when filter sterilized C & E mixed culture filtrate was added in 50% (v/v) concentration to 0.5% glucose fermentation medium which was then inoculated with culture #33. No such response was noted when the same concentration of culture filtrate was added from culture #33, or TC & E, or un-

inoculated D58 control medium filtrate. Significant differences between effect of sources of culture filtrate were shown to exist when tested at the .01 level of probability.

When selected levels of kinetin, instead of C & E culture filtrate, were added to the 0.5% glucose fermentation medium, culture #33 again demonstrated increased fermentative activity at several of the levels of kinetin tested. The response of culture #33 to the effect of kinetin followed a linear trend when tested at the .01 level of probability. However, at the .05 level of probability, these data were best fitted by a quintic degree of polynomial. This could most adequately be explained by the fact that more than one enzyme in the metabolism of glucose was responsive to the effect of kinetin.

Culture TC & E, the thermophilic partner in the C & E mixed culture, was not stimulated in its fermentation response by kinetin, at the sixteen levels tested. The kinetin trend analysis of TC & E data illustrated rather that the kinetin effect was following an inhibitory linear trend, when tested at the .01 level of probability.

When C & E culture filtrates from media containing yeast extract, kinetin, and/or sodium glycerophosphate were added to 0.5% glucose fermentation medium and inoculated with

culture #33, the following conclusions were drawn from the results: (1) 50% concentrations of filtrate were significantly better than 10% in stimulating fermentative ability of culture #33; (2) of the 50% concentrations of filtrates tested, filtrate from a medium containing both yeast extract and sodium glycerophosphate was found to be the most stimulatory; (3) the effects of filtrate from D58 (yeast extract) medium, and the kinetin-sodium glycerophosphate medium were not significantly different in demonstrating the enhanced fermentative activity of culture #33, when the data were tested at either the .01 level or the .05 level of probability. This finding indicates a resemblance of the factors in yeast extract to the combination of kinetin and sodium glycerophosphate, if not their identity.

LITERATURE CITED

Alexander, J. K.
1961 Characteristics of cellobiose phosphorylase. Journal of Bacteriology 81: 903-910.

Allen, M. B.
1950 The dynamic nature of thermophily. Journal of General Physiology 33: 205-214.

Allen, M. B.
1953 The thermophilic aerobic sporeforming bacteria. Bacteriological Reviews 17: 125-173.

Anderson, R. L. and E. E. Houseman
1942 Tables of orthogonal polynomial values excended to N = 104. Iowa Agricultural Experiment Station Research Bulletin 297.

Baker, H., O. Frank, I. Pasher, B. Black, S. H. Hutner, and H. Sobotka
1960 Growth requirements of 94 strains of thermophilic bacilli. Canadian Journal of Microbiology 6: 557-563.

Belasco, I. J.
1956 The role of carbohydrates in urea utilization, cellulose digestion and fatty acid formation. Journal of Animal Science 15: 496-508.

Braun, W.
1956 Cellular products affecting the establishment of bacteria of different virulence. New York Academy of Sciences Annals 66: 348-355.

Breed, R. S., E. G. D. Murray, and N. R. Smith
1957 Bergey's manual of determinative bacteriology. 7th ed. The Williams and Wilkins Co. Baltimore, Maryland.

Bullock, M. W., J. J. Hand, and E. L. R. Stokstad
1956 Syntheses of 6-substituted purines. American Chemical Society Journal 78: 3693-3696.

Campbell, L. L., Jr. and O. B. Williams
1953 The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. Journal of Bacteriology 65: 141-145.

Caplin, S. M. and F. C. Stewart
1948 Effect of coconut milk on the growth of explants from carrot root. Science 108: 655-657.

Castellani, A.
1926 Fermentation phenomena when different species of microorganisms are in close association. Society for Experimental Biology and Medicine Proceedings 23: 481-488.

Castellani, A.
1927 Symbiotic fermentation phenomenon: Its use in differentiation of microorganisms and identification of carbon compounds. Society for Experimental Biology and Medicine Proceedings 24: 511-516.

Conn, H. J. and M. J. Pelczar, Jr.
1957 Manual of microbiological methods. McGraw-Hill Book Company, Inc. New York, New York.

Cota, R. H.
1954 A study of the metabolism of aerobic mesophilic cellulose-decomposing bacteria. Dissertation Abstracts 14: 5-6.

Dahl, L. G.
1955 The influence of some inorganic salts on the sporulation of a strain of Bacillus stearothermophilus. Physiologia Plantarum 8: 661-668.

Daly, J. W. and B. E. Christenson
1956 Purines. VI. The preparation of certain 6-substituted and 6,9-disubstituted purines. Journal of Organic Chemistry 21: 177-179.

Downey, R. J.
1962 Oxidative enzymes in subcellular structures of Bacillus stearothermophilus. Dissertation Abstracts 22: 2548.

Elion, G. B., E. Burgi, and G. H. Hitchings
1952 Studies on condensed pyrimidine systems. IX. The synthesis of some 6-substituted purines. American Chemical Society Journal 74: 411-414.

Ellis, R. J.
1955 The mechanism of cellulose decomposition by mesophilic aerobic bacteria. Dissertation Abstracts 15: 672-673.

El-Shazly, K., R. R. Johnson, B. A. Dehority, and A. L. Moxon
1961 Biochemical and microscopic comparison of in vivo and in vitro rumen fermentations. Journal of Animal Science 20: 839-843.

Enebo, L.
1949 Formation of reducing sugars in thermophilic cellulose fermentation. Acta Chemica Scandinavica 3: 975-981.

Enebo, L.
1951 On three bacteria connected with thermophilic cellulose fermentation. Physiologia Plantarum 4: 652-666.

English, J. and J. Bonner
1937 The would hormone of plants. I. Traumatin, the active principle of the bean test. Journal of Biological Chemistry 121: 791-799.

English, J., J. Bonner, and A. J. Haagen-Smit
1939 Structure and synthesis of a plant wound hormone. Science 90: 329.

Friedman, S. M.
1961 Thermostability and molecular differences in bacterial flagella. Dissertation Abstracts 22: 28.

Fry, K. A.
1961 Properties of flagella obtained from the facultative thermophile Bacillus coagulans strain 43P. Dissertation Abstracts 22: 28.

Georgi, C. E., W. Militzer, L. Burns, and J. Heotis
1951 On the existence of a cell granule in a thermophilic bacterium. Society for Experimental Biology and Medicine Proceedings 76: 598-601.

Georgi, C. E., W. Militzer, and T. S. Decker
1955 The organelle nature of a particle isolated from Bacillus stearothermophilus. Journal of Bacteriology 70: 716-725.

Gibson, T. and Y. Abdel-Malek
1945 The formation of carbon dioxide by lactic acid bacteria and Bacillus licheniformis and a cultural method of detecting the process. Journal of Dairy Science 14: 35-44.

Gyllenberg, H. H. G.
1951 Some aspects of the taxonomy of thermophilic bacilli. International Bulletin of Bacteriological Nomenclature and Taxonomy 1: 138-141.

Hajny, G. J., C. H. Gardner, and G. J. Ritter
1951 Thermophilic fermentation of cellulosic and lignocellulosic materials. Industrial and Engineering Chemistry 43: 1384-1389.

Hammerstrom, R. A., K. D. Claus, J. W. Coghlan, and R. H. McBee
1955 The constitutive nature of bacterial cellulases. Archives of Biochemistry and Biophysics 56: 123-129.

Hungate, R. E.
1946 The symbiotic utilization of cellulose. Journal of the Elisha Mitchell Scientific Society 62: 9-24.

Hungate, R. E.
1950 The anaerobic mesophilic cellulolytic bacteria. Bacteriological Reviews 14: 1-49.

Isotalo, A.
1951 Studies on the ecology and physiology of cellulose-decomposing bacteria in raised bogs. Acta Agraria Fennica 74: 1-106.

Jablonski, J. R. and F. Skoog
1954 Cell enlargement and cell division in excised tobacco pith tissue. Physiologia Plantarum 7: 16-24.

Ke, Y. H.
1964 Cellulose digestion by bacteria. Unpublished Ph.D. thesis. Library, Iowa State University of Science and Technology. Ames, Iowa.

Kennell, D. E.
1959 The effects of indoleacetic acid and kinetin on the growth of some microorganisms. Unpublished Ph.D. thesis. Library, University of California. Berkeley, California.

Kissman, H. M. and M. J. Weiss
1956 Kinetin riboside and related nucleosides. Journal of Organic Chemistry 21: 1053-1055.

Knight, B. C. J. G. and H. Proom
1950 A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. Journal of General Microbiology 4: 508-538.

Koffler, H.
1957 Protoplasmic differences between mesophiles and thermophiles. Bacteriological Reviews 21: 227-240.

Kulayeva, O. N. and I. P. Vorob'yeva
1962 The mode of action of kinetin on protein synthesis. Soviet Plant Physiology 9: 77-79.

Lansford, E. M., C. G. Skinner, and W. Shive
1958 Augmented inhibition by kinetin and related compounds on growth of pteridine-inhibited Lactobacillus arabinosus. Archives of Biochemistry and Biophysics 73: 191-197.

Levinson, H. S., G. R. Mandels, and E. T. Reese
1951 Products of enzymatic hydrolysis of cellulose and its derivatives. Archives of Biochemistry and Biophysics 31: 351-365.

Lord, T. H.
1959 Determinative bacteriology laboratory manual. 2nd ed. Burgess Publishing Co. Minneapolis, Minnesota.

Macfarlane, R. G., C. L. Oakley, and C. G. Anderson
1941 Haemolysis and the production of opalescence in serum and lecitho-vitellin by the alpha toxin of Clostridium welchii. Journal of Pathology and Bacteriology 52: 99-103.

MacLeod, R. A. and J. F. Murray
1956 Some factors affecting cellulose digestion by rumen microorganisms in vitro. Journal of Nutrition 60: 245-259.

Maruzzella, J. C. and J. G. Garner
1963 Effect of kinetin on bacteria. Nature 200: 385.

McBee, R. H.
1950 The anaerobic thermophilic celluloytic bacteria. Bacteriological Reviews 14: 51-63.

McBee, R. H.
1954 The characteristics of Clostridium thermocellum. Journal of Bacteriology 67: 505-506.

Militzer, W. and L. Burns
1954 Thermal enzymes. VI. Heat stability of pyruvic oxidase. Archives of Biochemistry and Biophysics 52: 66-73.

Militzer, W. and L. C. Tuttle
1951 Thermal enzymes. III. Apyrase from a thermophilic bacterium. Archives of Biochemistry and Biophysics 31: 411-423.

Miller, C. O.
1961 Kinetin and related compounds in plant growth. Annual Review of Plant Physiology 12: 395-408.

Miller, C. O., F. Skoog, F. S. Okumara, M. H. von Saltza, and F. M. Strong
1956 Isolation, structure, and synthesis of kinetin, a substance promoting cell division. American Chemical Society Journal 78: 1375-1380.

Oates, R. P., T. S. Beers, and L. Y. Quinn
1961 Induction of heat resistance by filtrates of thermophilic bacterial cultures. Bacteriological Proceedings 1961: 58.

Oates, R. P., T. S. Beers, and L. Y. Quinn
1963 Ecology of the symbiotic growth of a mesophilic bacterium at thermophilic temperatures. Bacteriological Proceedings 1963: 44.

Oda, M., C. Ikehara, and Y. Ito
1950 Studies on the thermophilic cellulose digesting bacteria. I. On search and isolation of thermophilic cellulose digesting bacteria. Journal of Fermentation Technology 28: 205-213.

Quinn, L. Y.
1949 Isolation and morphological study of a thermophilic cellulose digesting organism. Unpublished Ph.D. thesis. Library, Purdue University. Lafayette, Indiana.

Quinn, L. Y., R. P. Oates, and T. S. Beers
1963 Support of cellulose digestion by Clostridium thermocellum in a kinetin-supplemented basal medium. Journal of Bacteriology 86: 1359.

Quinn, L. Y. and P. A. Tetrault
1950 The isolation of an obligate thermophile that digests cellulose. Bacteriological Proceedings 1950: 18.

Reese, E. T., W. Gilligan, and B. Norkrans
1952 Effect of cellobiose on the enzymatic hydrolysis of cellulose and its derivatives. Physiologia Plantarum 5: 379-390.

Reese, E. T. and H. S. Levinson
1952 A comparative study of the breakdown of cellulose by microorganisms. Physiologia Plantarum 5: 345-366.

Sie, E. H., H. Sobotka, and H. Baker
1961a Factor converting mesophilic into thermophilic microorganisms. Nature 192: 86-87.

Sie, E. H., H. Sobotka, and H. Baker
1961b Factors inducing mesophilic bacteria to grow at 55C. Biochemistry and Biophysics Research Communications 6: 205-209.

Siu, R. G. H. and E. T. Reese
1953 Decomposition of cellulose by micro-organisms. Botanical Review 19: 377-416.

Skoog, F. and C. O. Miller
1957 Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Society for Experimental Biology Symposia 11: 118-131.

Snedecor, G. W.
1956 Statistical methods. 5th ed. Iowa State College Press. Ames, Iowa.

Strong, F. M.
1958 Topics in microbial chemistry. John Wiley and Sons, Inc. New York, New York.

Thompson, P. J.
1961 A thermophilic aldolase: purification and partial characterization. Dissertation Abstracts 22: 31-32.

Viljoen, J. A., E. B. Fred, and W. H. Peterson
1926 The fermentation of cellulose by thermophilic bacteria. Journal of Agricultural Science 16: 1-17.

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